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CHANGES IN INFLUENZA VIRUS ASSOCIATED WITH ADAPTATION TO PASSAGE IN CHICK EMBRYOS

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INTRODUCTORY.

Research on the viruses of epidemic influenza is primarily concerned with their relationship to the human disease. It may be important therefore to stress that with the possible exception of strains maintained solely by ferret passage, all experimental work has been carried out with strains which differ considerably from the parent "human" strains and which, as far as can be judged from the small amount of available evidence, are incapable of producing typical influenza in human subjects.

In this paper it will be shown that the virus present in human throat washings or in the amniotic fluid of chick embryos infected with filtrates from these washings, differs sharply from the virus obtained in chick embryos after one or two normal passages. A method is also described for maintaining the "unadapted" character through at least a limited number of chick passages. The success of this method depends on, and provides a demonstration of, the fact that the change from the "unadapted" to the "adapted" state is a discontinuous mutation in the genetic character of the virus.

The results of an investigation of a typical influenza A epidemic in military camps during May, 1942, have recently been reported (Burnet *et al.*, 1942). In this work virus was isolated from filtrates of throat washings by the amniotic inoculation of chick embryos according to the method described by Burnet (1940). With the development of the Hirst (1941) red cell agglutination technique a simple method of detecting the presence of influenza virus in embryonic fluids was available. In addition to examining the cellular content of tracheal fluid (the most reliable index of infection in the earlier method) both amniotic and allantoic fluids were tested against fowl red cells. Positive agglutination results were obtained with amniotic fluids but it was immediately evident that the agglutination was weak and atypical in character. Allantoic fluids with two exceptions gave no trace of agglutination in these embryos inoculated with the original throat filtrates. On the assumption that strains of virus just isolated from human tissues might agglutinate mammalian cells more readily than the standard fowl cells, Dr. W. I. B. Beveridge suggested the use of human and guinea-pig red cells. These were known to be susceptible to agglutination by influenza viruses from the work of McLelland and Hare (1942) and a more extensive study by Clark and Nagler (1943) in this laboratory had shown that cells of these two species were agglutinated more consistently and to higher titres than any other available mammalian cells. Tests with fluids from embryos infected with human material showed in fact that amniotic fluids gave much higher titres with both types, guinea-pig cells being somewhat more susceptible than human. The allantoic fluids however showed no more evidence of agglutination than with fowl cells. We know that all our stock influenza virus strains agglutinated fowl cells to a slightly higher titre than guinea-pig cells and it became evident that a well defined characteristic of freshly isolated virus

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had been discovered. This paper records the results of an attempt to define and assess the significance of this difference between freshly isolated and stock influenza virus strains.

TECHNIQUE.

Hirst haem-agglutination titrations. The technique used in Hirst agglutination tests in this laboratory has previously been described (Burnet, 1942) and only a brief account will therefore be given here. Two sets of doubling dilutions of the fluid are made from 1: 10 to 1: 320 in saline, using a volume of 0·25 ml. Another similar volume of saline is added to each tube and then 0·25 ml. of washed fowl or guinea-pig cells in 2 p.c. suspension. The tubes are shaken, placed in the refrigerator, and read as soon as the cells have deposited completely. The end point is taken as the dilution showing an arbitrarily chosen degree of incomplete agglutination. This "+" value is given when there is a compact central "button" of unagglutinated cells surrounded by openly distributed granular aggregates easily visible with the naked eye. The appearances with fowl and guinea-pig cells differ somewhat in character, the "+" guinea-pig cell reaction showing finer granules and less open space between them than the fowl cell reaction. The character of the deposit changes rapidly around the end point and it is easy to interpolate a value for the end point when it lies between two tubes. Throughout this paper much use will be made of the ratio between the fowl and guinea-pig cell titres as determined in this fashion; the values are expressed as F/G ratios, a typical "unadapted" value being 15/160.

It should be noted that the dilutions stated in such ratios are those of the primary virus dilution to which equal volumes of saline and of red cell suspension are subsequently added. The final dilutions are therefore three times those stated.

When antibody titrations by Hirst's method were required fowl cells were used wherever possible but with unadapted strains guinea-pig or human cells were used. Human cells (group O) are needed if antibody in human sera is to be titrated as most human sera agglutinate guinea-pig cells.

Titration of virus and antibody in ovo. Titrations of fluids for virus content were made using either the amniotic or the allantoic methods of inoculating 12 day chick embryos. Tenfold dilutions were prepared in ice-cold saline containing 10 p.c. of normal horse serum and quantities of 0·05 ml. inoculated. Amniotic inoculations were made by the standard method (Burnet, 1940). The method of allantoic inoculation described by Burnet (1941) has been still further simplified (see Burnet and Beveridge, 1943) and is now as follows: The required number of fertile eggs is marked at a suitable spot and a small drill cut made through the shell. A swab dipped in sterile molten paraffin wax is brushed over the area immediately surrounding the drill hole to provide a sterile non-wetting surface on which a drop of inoculum can be placed. The eggs are then labelled and placed with the drill hole uppermost. A straight surgical needle is pushed about 3–4 mm. into the allantoic cavity and as it is being withdrawn a measured 0·05 ml. of inoculum is deposited at the puncture by means of a calibrated capillary pipette. Gravity and the slight negative pressure (from cooling) in the air space cause the drop of fluid to run immediately into the allantoic cavity. All eggs are opened after three days further incubation at 35° C. and the presence or absence of specific infection is determined by making a rough agglutination test with the amniotic or allantoic fluid and whichever type of red cell is most appropriate to the circumstances.

Antibody titrations by the allantoic method followed the same technique; mixtures of equal volumes of suitable dilutions of virus and serum being inoculated in 0·05 ml. volumes. Results are expressed as the logarithm of the number of 50 p.c. infective doses inactivated by undiluted serum. Details of technique and a discussion of the type of result obtained by this method will be found in a recent paper by Burnet and Beveridge (1943).

Reisolation of virus from infected ferrets. Nasal washings from infected ferrets were obtained by lightly anaesthetizing the animal and holding it inverted with the nares dipping into 15 ml. of broth in a tilted petri dish. As the animal emerges from the anaesthetic it tends to breathe in through the mouth and out through the nostrils so that a good deal of material from the nasal cavity is washed into the broth. Needless to say the method must be varied according to the individual reaction of each ferret, e.g. by having an assistant close the animal's jaws occasionally if it refuses to breathe through the nose at all. It is advisable for operator and assistant to be insusceptible to the virus being studied as there is inevitably heavy droplet contamination of the atmosphere. The washings are filtered through paper and then through a gradocol membrane. This technique of washing out the ferret's nose, and inoculating the filtrate amniotically, offers a means of studying ferret influenza more closely and in particular makes it possible to use a ferret both for the passage of virus and for the production of the corresponding immune serum—a desirable combination in many types of experiment.

Inhibitory effect of human tears. For the experiments with tears, fluid was obtained by means of capillary tube and teat from the outer canthus with the head so turned that the inner canthus was vertically above the outer. Some subjects produced sufficient secretion without further stimulus, others needed the slight degree of irritation obtained by exposing the conjunctiva to the

vapour of chloracetophenone (tear gas C.A.P.). Usually tears from several individuals were pooled and the pooled fluid heated at 56° C. for 30 minutes before being stored in the refrigerator. Tests with various types of virus were made essentially by the technique used in serum neutralization tests, with the modification that the virus-diluted tears mixtures were left in contact in the refrigerator overnight before adding red cells.

THE BEHAVIOUR OF UNADAPTED VIRUS ON AMNIOTIC PASSAGE.

As previously reported all the primarily infected amniotic fluids obtained from eggs inoculated with filtrates of human throat washings from the May, 1942, epidemic, as far as they were tested, gave low F/G ratios (Burnet *et al.*, 1942). In the ordinary routine, subinoculations were made with undiluted amniotic fluid and whenever tested for the F/G ratio the second passage amniotic fluids gave a value close to unity. In the course of an experiment to determine approximately the content of virus in these primary amniotic fluids it was noted that eggs inoculated with high dilutions gave amniotic fluids with a low F/G ratio. These eggs also gave no evidence of virus in the allantoic fluids.

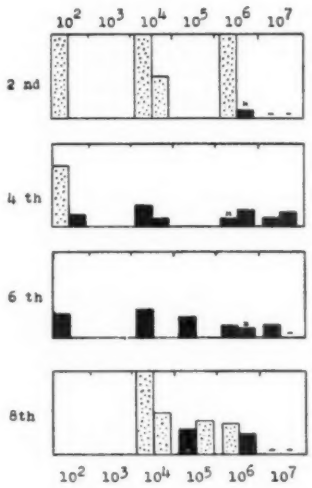

Fig. 1. Diagram to show distribution of F/G ratios in 2nd, 4th, 6th and 8th passages of first passage series of BEL.

Each vertical bar represents the F/G ratio of an infected fluid obtained by inoculating the corresponding dilution of an O type fluid of the previous passage. O type fluids ratio 0·3 or less are black, D type stippled. An asterisk indicates the fluids used for passage.

In order to follow up this observation, amniotic passages of the strain were made according to two different methods. In the first series fluids from eggs inoculated with high dilutions and showing low F/G ratios were used for transfer at dilutions of 10^{-5}, 10^{-6} and 10^{-7}. In the second series after two pasages at low dilution (1: 100) a full series of dilutions was tested. Table 1 contains the significant results from this experiment which provided the general pattern for subsequent more detailed study of the phenomenon.

It will be seen that when limiting dilutions were used the character of the virus remained of the original type for three generations. With low dilutions the virus changed immediately to the type with an F/G ratio near unity and once this change had occurred all subsequent passages whether at limiting dilutions or otherwise gave fluids showing high F/G ratio.

It was impractical to carry out detailed titration passages of this sort on all strains, but since primary amniotic fluids in every instance showed a low F/G ratio and the fluid from at least one subsequent passage of five of the eight strains isolated gave the standard high F/G ratio, it is reasonable to assume that all would have shown essentially the same behaviour as those tested in detail. In the laboratory we have referred to strains showing a ratio of less than 0·25 as "unadapted" and those giving a value above 0·5 as "adapted", but as this is liable to lead to confusion with the commoner usage of "adapted" for strains showing full pathogenicity for some laboratory host we shall refer to them here as "original" (O) and "derivative" (D) types respectively.

When the general character of the changes with passage had been recognized, attempts were made to maintain a strain in the "original" state through an extended series of passages. First passage fluids maintained in a solid CO_2 refrigerator were used as starting points and after failing with three strains, two satisfactory series of passages were initiated with the strain BEL. In the first series a ferret, F174, was inoculated intranasally with the first passage material and on the second, third, sixth and eighth day after inoculation nasal washings were obtained. Filtrates

were prepared from these and titrated by the amniotic method with the results shown in Table 3. It will be seen that the "original" type of behaviour is shown by the limiting dilutions of each positive filtrate.

TABLE 1.

Comparison of the results of amniotic passage of strain DOW (a) at low dilutions, (b) at limiting dilutions.

1st			Primary amniotic fluid 62311			
2nd	Undil.	62430	150/160	$1:10^6$	62438	50/320
3rd	1:100	62590	160/200	$1:10^6$	62595	5/100
4th	$1:10^7$	62843	80/50	$1:10^8$	62849	30/160
5th				$1:10^7$	63079	80/80

For each passage are shown (1) The dilution at which the fluid shown in the line above was inoculated by the amniotic route; (2) Serial number of the egg used; and (3) F/G ratio of the amniotic fluid.

TABLE 2.

Reisolation of unadapted virus from ferret nasal washings by amniotic inoculation.

	Dilution of nasal washing filtrate.											
	Undiluted.				10^{-1}.				10^{-2}.			10^{-3}
2nd day	+ + + +	220/320	25/60		+ + + +	12/32	240/160		+ + + − −	80/640*	70/360	− −
3rd day	+ + +	120/360	140/200						+ − −	120/320		
6th day	+ + −	80/640	80/640									
8th day	− − −											

+ Amniotic fluid showing positive agglutination. − Amniotic fluid not agglutinating red cells. F/G ratios shown for representative fluids.

From the fluid indicated by an asterisk in Table 2 serial dilutions were prepared and eggs were inoculated amniotically with dilutions 10^{-2}, 10^{-4}, 10^{-6} and 10^{-7}. One of the eggs inoculated with the 10^{-6} dilution gave amniotic fluid with a low F/G ratio and a similar series of dilutions from this fluid was tested amniotically. In this way passage of unadapted type was maintained for eight generations. The essential features of this passage series can be seen from the graphic representation of the second, fourth, sixth and eighth passages in Fig. 1. The shaded columns represent the value of the F/G ratio. The figure shows *first* how in each passage except the sixth a proportion of fluids containing derivative type virus (F/G more than 0·5) is found in the lower dilutions, and *second* how the proportion of "original" fluids was greatest at fourth, fifth and sixth passages and much more strictly limited to the highest dilutions in passages before and after these. The passage series was terminated at the ninth generation when all fluids showed an F/G ratio greater than 0·3.

TABLE 3.

Passage of derivative strains of BEL.

	Amniotic series.				Allantoic series.		
Passage.	Dilution.	No.	F/G ratio.	Passage.	Dilution.	No.	F/G ratio.
2		67815	320/360	2		67815	320/360
3	10^{-4}	68059	320/320	3	10^{-4}	68006	160/160
4	10^{-4}	68114	360/480	4	10^{-4}	68247	240/240
5	10^{-4}	68498	320/320	5	10^{-6}	68508	150/120
6	10^{-6}	68723	280/360	6	10^{-4}	68726	200/160
7	10^{-4}	69033	100/120	7	10^{-4}	69039	160/140
8	10^{-2}	69244	200/200	8	10^{-2}	69247	300/140
9	10^{-2}	69374	1000/1000	9	10^{-2}	69376	320/160
10	10^{-2}	69439	1000/1000	10	10^{-2}	69444	160/160
11	10^{-5}	69531	480/240	11	10^{-2}	69534	160/80

Note. At each passage the dilution indicated is that at which the fluid shown in the previous line was inoculated.

A second series was started directly from the stored first passage fluid which when titrated amniotically gave two "original" type fluids at dilutions 10^{-2} and 10^{-4} from the second of which (67809) the "original" ("O") passage series was begun. Fluids from the 10^{-2} dilution gave

''derivative'' ratios and one of these (67815) was chosen as starting point for a parallel ''derivative'' (''D'') series of passages. The O series was almost lost after two further passages because all fluids gave D ratios when the eggs were opened after three days incubation. On retitrating the fluid and opening eggs after two days an O type fluid was obtained (68303) from which passage could be continued. Fig. 2 shows graphically the results obtained over ten passages during which O type fluids were obtained from some at least of the higher dilutions.

At the same time two series of ''derivative'' passages were made using at each passage limiting dilutions and inoculating one series amniotically and the other allantoically. Table 3 shows the F/G ratios of the fluids used for each passage in these series. At each passage several other fluids were tested against fowl and guinea-pig cells and in sharp contrast to the results in the O series all gave similar ratios to those shown in the Table. The average values were lower as it was our practice to select for passage the fluid with the highest agglutination titre.

COMPARISON OF ORIGINAL AND DERIVATIVE TYPES OF STRAIN BEL.

As the work developed it was found that an increasing number of differences in behaviour could be correlated with the differences in F/G ratio which we have used as the primary criterion for dividing virus strains into original and derivative types. The most clear cut and the most practically important is the failure of the O type to multiply after inoculation into the allantoic cavity. Other differences concern the average amount of amniotic fluid found in embryos inoculated amniotically, the infectivity and immunizing power of the strains when inoculated intranasally in mice and guinea-pigs, and the range of animal species providing red cells susceptible to agglutination by the viruses. Finally there are slight serological differences and a rather sharp difference in the degree to which the haem-agglutinating power is inhibited by human tears.

In considering the results of experiments on these points it is important to remember that O type virus can be obtained only in the form of amniotic fluid which always contains a variable amount of D type virus in addition. Virus in the D phase can be obtained either as amniotic or allantoic fluid and though absolute proof of the point is probably impossible, will normally be quite free of O type virus. For many of the experiments, pooled O type amniotic fluid was used, all suitable fluids from one set of titrations being pooled and one-third of the volume of pure glycerol being added. When stored in the refrigerator such virus retained its characteristic F/G ratio almost unchanged for some months.

TABLE 4.

Comparative titrations of ''original'' type amniotic fluids.

Dilution.	Amniotic titration.				Allantoic titration.			
Amniotic fluid 65620								
10^{-7}	12/120	0/25	—		—	—	—	—
10^{-6}	35/160	5/50	+		—	—	—	
10^{-5}	+	+			—	—	—	—
10^{-4}	40/160	40/640			—	—	—	—
10^{-3}					0/1	0/5	10/30	—
10^{-2}	30/40	+			30/30	160/120	140/160	60/80
Amniotic fluid 67809								
10^{-7}	30/240	0/5	—	—				
10^{-6}	40/160	8/40	280/280	—	—	—	—	
10^{-5}	480/400	240/320	400/320		100/80	120/120	—	—
10^{-4}					200/160	480/100	+	+

F/G ratios are shown for most infected embryos, + signifies that virus was proved present but no titration for F/G ratio was done, — fluid gave no agglutination.

Failure of O type to grow in the allantoic cavity. It has previously been reported that as a rule no evidence of virus multiplication was obtained when throat filtrates from patients were inoculated allantoically (Burnet *et al.*, 1942) although they were known to be infective by the

amniotic route. When an O type amniotic fluid is titrated both by amniotic and allantoic inoculation a consistently higher titre is obtained by the former method and with only minor deviations the titre determined by allantoic titration corresponds with the titre to which D type fluids are obtained in amniotic titration. The difference between the two end points varies from fluid to fluid. Two typical examples of such double titrations are given in Table 4. This Table may be summarized as indicating that fluid 65620 contained approximately 10^7 infective units of O type virus and only 100 to 1,000 units of D type. A particularly interesting feature of this titration is the suggestion that in the eggs inoculated allantoically with virus diluted 10^6 a development of "derivative" virus was just beginning when the eggs were opened on the third day. Fluid 67809 also contained approximately 10^7 infective units of O type but a larger proportion (10^5) of D type.

These results are characteristic of those obtained in other titrations. It should be stressed that in an experience of several hundred F/G titrations of allantoic fluids we have never obtained a typical O ratio. Very rarely low titre fluids such as are shown in the Table are encountered but whenever a normally high titre is obtained the F/G ratio is 0·5 or higher. A point not shown in the results tabulated is the occasional appearance of a D type fluid from a dilution which would be expected to give only O type infections. One or two examples of this may be seen in Fig. 2.

When D strains are similarly titrated by both methods there is no significant difference between the end points and all fluids are of the D type. Titres as a rule are higher than those of O type fluids, a proportion of eggs inoculated with 10^{-8} dilutions becoming infected. Table 5 shows some typical results.

TABLE 5.

Comparative titrations of D type substrains of BEL.

Fluid.		Dilutions. 10^8	10^7	10^6
66480 (8th passage)	Amniotic titr.	+ + + +	+ + + +	+ + − − −
	Allantoic „	+ + + + −	+ + + + +	+ + + + −
67815 (2nd passage)	Amniotic titr.	+ + +	+D + + +	+D +D + −
	Allantoic „	+ + +	+ + + +	+D +D +D −

Variations in the amount of amniotic fluid after three days incubation. In the course of these titration passages it was noticed that many of the amniotic fluids with the typical low F/G ratio were in larger amount than normal. An approximate statement of the volume of each amniotic fluid was made as the eggs were opened and these values were later correlated with the F/G findings. If we confine attention to titrations giving either O type alone or both O and D type fluids the distribution of amniotic fluid volumes is as shown in Fig. 3. This ensures that only recently developed D strains are included. After repeated amniotic passage in the D state the amount of amniotic fluid found at three days is very often less than 0·1 ml. This is a general characteristic of influenza virus strains adapted to amniotic passage and was described in the first communication on the method (Burnet, 1940).

Fig. 3 shows that O type infections result in a considerably increased production of amniotic fluid above that observed in eggs inoculated with noninfective material while D type infections show a predominance of small volumes. These differences hold equally for both the early and later passages of the series.

A considerable number of embryos known to be infected with O or D type virus were studied histologically by Major W. Wartman, M.C., U.S. Army. Lesions with both types of infection were practically limited to the lungs. Individual embryos varied in the degree to which the lung damage had developed in two and three days but there was no systematic difference between the two types of infection. The histological picture conformed throughout to that described by **Burnet (1940).**

Nothing was observed which could suggest any reason for the differences in the amount of amniotic fluid.

Agglutination of different red cell species by O and D type strains. Most of the easily available types of red cell have been tested against O and D type amniotic fluids. Table 6 shows the results obtained. The Table may be summarized as showing that human and guinea-pig cells are approximately equally susceptible to both types, ferret cells fall into the same category but have a much lower susceptibility to both. All other types of cell are much more readily agglutinated by "derivative" than by "original" strains. Mouse cells have proved useful for some types of experiment as they show a high titre with D strains and no agglutination with most O fluids.

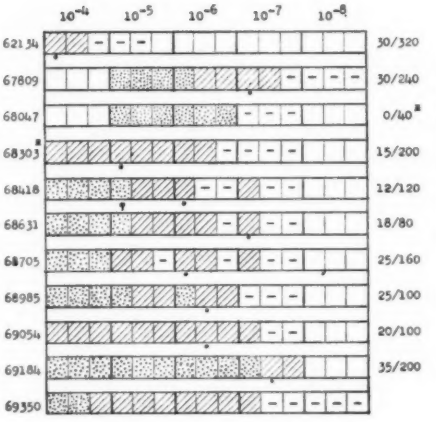

Fig. 2. Diagrammatic representation of second passage series of BEL.

Each horizontal bar represents the result of a titration of the amniotic fluid whose number is prefixed. Each stippled square represents a D type infection, each cross hatched square an O type infection. A blank indicates that no inoculation was made and a minus sign that the corresponding embryo was not infected. A dot below a square indicates that this fluid was used for the next passage; its F/G ratio is shown at the right. * Fluid 68303 was obtained from a repetition of the titration of 68047 (see text).

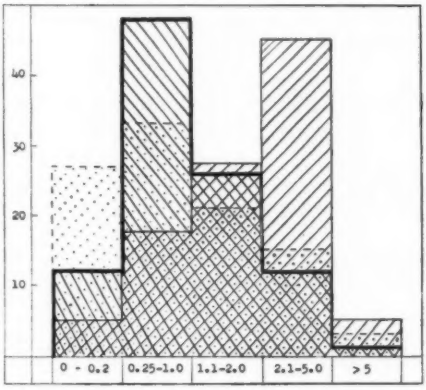

Fig. 3. To show amounts of amniotic fluid in uninfected embryos and in embryos infected with O and D type virus.

Heavy line with diagonal shading downward to the right shows the distribution of amniotic fluid volumes in uninfected embryos, light unbroken line with diagonal shading to the left in infected embryos giving O type fluids, and broken line with stippling in infected embryos giving D type fluids. Ordinates percentage of fluids in each group showing the volumes in ml. indicated as abscissae.

Human (group O) cells are essential if one requires to titrate human sera with O type virus as guinea-pig cells are agglutinated by a large proportion of human sera. For general purposes and for immunological experiments with ferret or mouse sera we have found guinea-pig cells more satisfactory to work with.

TABLE 6.

Activity of O and D types of BEL on different red cells.

Species of red cell.	68417 (O).	66187 (O).	68731 (D).	68724 (D).
Fowl	25	30	140	160
Guinea-pig	120	120	40	160
Human		100		150
Ferret	15		10	
Cow	<10, <10	<10, <10	<10, 20	<10, <10
Sheep	<10	<10, <10	50	120, 50
Goat	<10, <10, <10	<10, <10	35, 35, 100	60, 25
Horse	<10	<10	10	<10
Mouse	<10	<10		120

Note. Where more than one value is given, cells from different individuals of the same species were tested.

Agglutination tests using Hirst's standard method. Since our routine method of carrying out haem-agglutination tests differs considerably from that described by Hirst (1942), we have examined typical O and D fluids by his method. The tests were kindly done for us by Miss D. Lush who had had considerable experience with the standard Hirst test at Hampstead. The pooled

amniotic fluids used as O virus contained 33 p.c. of glycerol which interfered somewhat with the readings of the lowest dilutions. Otherwise the technique corresponded strictly to that recommended by Hirst. Table 7 gives the readings.

TABLE 7.

Comparative titration of O and D strains of BEL by Hirst's standard method.

		Final virus dilutions.									
		2	4	8	16	32	64	128	256	512	Titre.
Fowl cells											
BEL	O	(+)	++	±	—	—	—				4
BEL	D					+++	+++	++	+	—	128
Guinea-pig cells											
BEL	O		+++	+++	+++	++	±				32
BEL	D			+++	+++	++	+	—			32

Selective absorption with fowl and mouse cells. From Table 6 one would deduce that by absorbing a mixture of O and D virus with either fowl or mouse cells the supernatant should show a diminished proportion of D virus.

The following experiment illustrates the changes in Hirst agglutinations produced by absorption of O and D strains of BEL with fowl and guinea-pig cells. Serial twofold dilutions in saline of O and D type fluids of strains BEL were prepared, ranging from 1 : 10 to 1 : 640, each having a total volume of one ml. Three such sets were prepared for each strain, to one set 0·5 ml. of 2 p.c. fowl cells, to the second 0·5 ml. of guinea-pig cells and to the third 0·5 ml. of saline were added. The tubes were placed in a refrigerator, shaken again after an hour and then left overnight in the cold. From each tube 0·5 ml. of supernatant fluid was transferred to corresponding tubes in two similarly arranged racks. When these transfers were completed one rack received 0·25 ml. of 2 p.c. guinea-pig cells to each tube while fowl cells were similarly added to the other. Readings were made as usual after two hours in the cold. (Table 8).

TABLE 8.

Absorption of O and D type virus with fowl and guinea-pig red cells.

Virus and treatment.	Tested against.	Initial virus dilutions. 10	20	40	80	160	320	640
O-type virus untreated	F.	++	++	+	tr	—	—	—
" " "	G.	+++	+++	+++	+++	++	+±	tr
" " absorbed F.	F.	—	—	—	—	—	—	—
" " " F.	G.	+++	+++	++	+±	±	—	—
" " " G.	F.	—	—	—	—	—	—	—
" " " G.	G.	—	—	—	—	—	—	—
D-type virus untreated	F.	+++	+++	++	++	+±	±	—
" " "	G.	++	++	++	++	+±	±	tr
" " absorbed F.	F.	±	—	—	—	—	—	—
" " " F.	G.	+	tr	—	—	—	—	—
" " " G.	F.	+±	tr	—	—	—	—	—
" " " G.	G.	—	—	—	—	—	—	—

F = fowl red cells; G = guinea-pig red cells. See text for details of experiment.

With mouse cells it is possible to divide a typical "original" fluid into relatively pure O and D fractions by making use of the secondary elution of virus from red cells recently described by Hirst (1942). The following experiment is typical.

The virus used was a 10 p.c. dilution in saline of a pool of typical O amniotic fluids. To 1 ml. of this dilution 0·25 ml. of various concentrations of mouse red cells were added in the cold and the mixtures left overnight in a refrigerator. Next morning the supernatant fluids were removed for titration and the cells washed once with cold saline. One ml. of saline was then added, the cells emulsified and left with occasional shaking for 5 hours in an incubator at 35° C. At the end of this time the tubes were centrifuged and the supernatant fluid containing the eluted virus also removed for titration.

TABLE 9.

Absorption of O type fluid with mouse cells.

Final concentration of mouse r.b.c.'s.	Absorbed fluid.	Eluate.
10 p.c.	5/160	12/35
5 p.c.	5/240	15/30
1·25 p.c.	10/360	±5/12
0·62 p.c.	16/400	tr
Unabsorbed	32/400	—

F/G ratios are shown. For details see text.

Table 9 shows the F/G ratios of the absorbed fluids and of the eluates from the red cells used. The chief interest of this Table is its suggestion that pure O type virus may be completely inactive against fowl cells, the agglutination normally found representing merely the effect of variable amounts of D virus which is always present.

This conclusion however does not seem to be compatible with some of the results of amniotic titration which indicate that some fluids contain very much less D type virus than the 5–20 p.c. which would be indicated on this view by their F/G ratio. In all probability the absorption results merely indicate a considerable degree of heterogeneity amongst the virus particles grouped together as O, some having considerably higher powers of adsorption to fowl red cells than others.

Serological differences between O and D types. A study of the antibody content of sera from ferrets inoculated with D and predominantly O material has shown that small but definite serological differences between the two phases can be recognized. It will be convenient to base most of the discussion on two ferret sera, F179 and F180, specially prepared for the investigation and to note later the confirmation afforded by other ferret sera from animals inoculated for various reasons with virus from the same epidemic.

Ferret 180 was inoculated intranasally with O type virus diluted 1: 10^5 (66187 BEL). In an amniotic titration done at the same time this dilution gave two infections (O type) and two failures to infect; one of four eggs inoculated at 1: 10^6 also gave a positive (O) result. The ferret was therefore inoculated with the nearly limiting infective dilution of a predominantly O fluid and in all probability only O type virus particles were instilled into the nose. Ferret 180 showed a rise of temperature to 104·0° F. on the fourth day and on the fifth day washings from its nose were filtered and tested. The undiluted filtrate gave infections in all eggs with amniotic fluids giving F/G ratios of 15/120, 20/100, 150/500, i.e. two of O type, one intermediate in character. This confirms the "original" character of the infection.

Ferret 179 was similarly inoculated with the well adapted allantoic fluid strain 66480, F/G 300/200, at a dilution of 10^{-5}. The fluid titrated amniotically gave two out of four infections at 1: 10^5 both of fully adapted type, and all eggs at lower dilutions were also infected. The ferret showed only a weak rise in temperature (103·4° maximum) but developed a satisfactory antiserum. From both animals a preliminary bleeding was made and the serum shown to contain no significant antibody. The ferrets were bled to death under anaesthesia fifteen days after inoculation.

TABLE 10.

Hirst titrations of F179 and F180 against the virus stocks used for immunization.

		Serum dilutions.						
Serum.	Virus.	100	200	400	800	1,600	3,200	Titre.
F179	D/50	—	—	—	—	+±	++	1,300
(D)	O/20	—	—	—	+	++	++	800
F180	D/50	—	—	—	+±	++	++	650
(O)	O/20	—	—	—	—	+±	++	1,300

Guinea-pig cells were used throughout.

For obvious reasons antibody titrations relevant to the present problem could not be made by the standard methods in mice or on the chorioallantois or by Hirst's method using fowl cells as indicator. The sera were tested against numerous virus preparations using the modified Hirst technique described earlier with guinea-pig instead of fowl cells. A few tests were also made of the power of the sera to inactivate O or D type virus *in ovo*. Mixtures of serum with O type virus were inoculated into the amniotic cavity, D type into the allantoic. Tables 10 and 11 show the results of these titrations. It will be seen that there is a clearly demonstrable advantage in Hirst tests with the homologous strains. In using these sera to characterize different virus samples

serologically we have adopted the convention of using the ratio: Titre with D serum (F179)/Titre with O serum (F180). The homologous strains would, from the experiment tabulated, give serological ratios of, for the D strain 66480 1300/650 = 2·0, and for the O strains 66187 800/1300 = 0·6. In testing other virus strains the same general set-up is used both sera being diluted serially from 1: 100 and the virus diluted to a convenient value (five times the concentration giving end-point agglutination of guinea-pig cells).

TABLE 11.

Neutralization tests in ovo *with sera F179 and F180.*

	F179 (D).	F180 (O).
A. Against D virus 66480, allantoic inoculation.		
S + V	− − − −	− − − −
S/5 + V	− − − −	− − − −
S/25 + V	+ + + +	+ + + +
B. Against O virus 66187, amniotic inoculation.		
S/5 + V/10	− − −(−)	− − − −
S/25 + V/10	+ − − −	− − − −
S/50 + V/10	+ + + +	+ + + −

The neutralization tests *in ovo* also indicate that the sera are of approximately equal activity and they show very little evidence of greater action on the homologous strain. However much larger numbers of eggs would be needed to establish a difference of the same order as that found in the reciprocal Hirst tests and the results shown are quite compatible with the existence of such a difference.

In no instance have we observed any greater differences in cross neutralization tests than the approximate twofold advantage to the homologous serum-virus pair shown in Table 10. On some occasions there has been only a slight advantage and there has been a tendency for the differences to become less marked as the sera age. The mean values for the ratios D/O have been for thirteen O type fluids 0·73 (0·5–0·92) and for fifteen D type fluids 1·10 (0·83–2·0). Attempts to accentuate these differences by absorption of the sera in various ways have been to a certain extent successful but it was not possible to obtain satisfactorily reproducible results and the matter did not seem of sufficient importance to warrant an extensive investigation.

Other attempts to detect serological differences were made by immunization of rabbits and guinea-pigs with appropriate material. Rabbits were immunized intravenously with living virus. High titre sera were obtained but there was no evidence of specificity in haem-agglutination tests.

Intranasal infection of guinea-pigs. Guinea-pigs were immunized by intranasal administration of virus. In confirmation of Stuart Harris (1937) we found that although no symptoms were observed well marked antibody-production occurred. Three animals were inoculated with each type of fluid, a few drops being run into each nostril under light ether anaesthesia. The inoculations were repeated 24 hours later. Blood was obtained before inoculation by heart puncture and the animals were finally bled three weeks later. Titrations were made by modified Hirst methods against O and D strains and by allantoic inoculation against the homologous D strain. (Table 12). The results show no evidence of serological specificity but there is, by both methods, a significantly higher antibody production in guinea-pigs given the O type virus The Hirst tests have a level approximately twice that of the animals given type D while in the allantoic tests there is a 25 fold advantage.

TABLE 12.

Antibody response of guinea-pigs inoculated intranasally with O and D phases of BEL.

		Hirst titration.		Allantoic titration.
Guinea-pig	Inoculated with	O	D	D
11	O	320	240	5·8
12	O	400	280	8·0
19	O	360	320	7·8
Average		360	280	7·2
13	D	160	120	6·5
14	D	200	180	5·5
20	D	140	120	5·3
Average		170	140	5·8

The ability of O and D type virus to infect and immunize mice. In view of the fact that the mouse inoculated intranasally is currently regarded as the standard experimental animal for influenza virus studies, we have paid considerable attention to the effect of various substrains of BEL given by this route. All the O type strains which have been tested at a dilution of 1: 10 have failed to produce any visible lesions. Since these strains must of necessity be used in the form of amniotic fluid, which always contains a high protein concentration and is frequently viscous, tests with undiluted fluid were unsatisfactory and were not normally made. D type strains in the form of allantoic fluid gave extensive consolidation and occasionally death when inoculated undiluted, small grade 1–2 lesions were produced at 1: 10 and usually nothing at 1: 100.

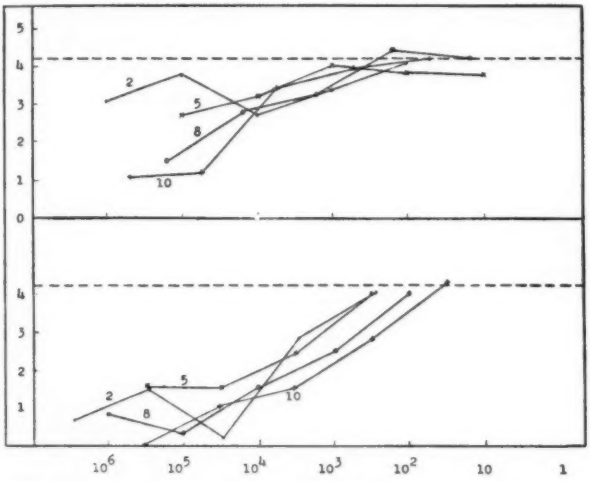

Fig. 4. Immunization of mice with O and D type virus.
Ordinates show the average grade of lung lesion observed in each group of mice after they had been tested for immunity. Control mice gave values approximating 4·2. Abscissae indicate the number of 50 p.c. infective doses for the chick embryo inoculated in the immunizing dose. In the upper section the results with 2nd, 5th, 8th and 10th passage O strains, in the lower section the corresponding results with D strains are shown.

In previous work we had found that strains which had undergone only a few amniotic passages although they produced only slight consolidation were capable of immunizing mice to a relatively high titre when given intranasally. On the basis of this result we suggested (Burnet and Foley, 1940) that virus in human throat washings might be detected by inoculating mice and subsequently testing them for immunity. Two positive results were obtained in 1940 but experience in 1942 failed to conform the usefulness of the method. It was hoped that a detailed study of the immunizing capacity of a virus strain in both O and D phases would indicate the reasons for this irregularity. Experiments were therefore carried out with fluids of 2nd, 5th, 8th and 10th egg passages in both O and D phases of strain BEL. The 5th and 8th passage experiments were done with the first series of passages (referred to earlier) and the 2nd and 10th passage experiments with the second series. Table 13 gives a summary of the history of each strain used, H representing the original human filtrate, F passage through a ferret, O amniotic passage in the O phase, D amniotic or D* allantoic passage in D phase.

TABLE 13.

History of strains used for mouse immunization.

2nd passage	O	67809	H O O
	D	67815	H O D
5th „	O	65717	H O F O O O O
	D	65770	H O F O O D D
8th „	O	66187	H O F O O O O O O O
	D	66480	H O F O O D D D D D
10th „	O	69350	H O O O O O O O O O O
	D (amn.)	69531	H O D D D D D D D D D
	D (all.)	69534	H O D D* D* D* D* D* D* D* D*

The type of passage is indicated as follows: H original human material; O amniotic inoculation in the O phase; D amniotic inoculation in the D phase; D* allantoic inoculation in the D phase; F intranasal passage in ferrets.

Technique. Serial tenfold dilutions of virus-containing fluid were made in ice-cold, horse-serum saline, and titrations made by both amniotic and allantoic inoculation methods for O type fluids and by the allantoic method for D fluids. Immediately afterwards six mice were inoculated intranasally with each dilution from 10^{-6} to 10^{-2} for D strains, from 10^{-5} to 10^{-1} for O strains. Three weeks later all mice were reinoculated with standard mouse passage "Melbourne" virus diluted 1: 100 from stock 10 p.c. mouse lung suspension. Strain BEL is serologically very close to "Melbourne" and in adequate dose produces a high degree of immunity to the latter. The test dose killed about 90 p.c. of control mice within seven days; any survivors had very extensive consolidation of the lungs. Experimental and control mice were examined at death and survivors killed seven days after the test inoculation. The degrees of pulmonary consolidation were recorded according to the usual convention 0–4, while mice dying specifically in four or five days were given the value 5. For each group the average value was calculated and used in plotting the results shown in Fig. 4. The 50 p.c. end point of infectivity for chick embryos was also calculated from the results of amniotic or allantoic titrations. In the Figures the immunizing dose given is presented in terms of the 50 p.c. infective dose *in ovo*. Table 14 combines the titration data and in the final column shows the number of 50 p.c. infective doses needed to immunize mice sufficiently to give an average grade 2 lesion (about ½ consolidation at 7 days).

Two well defined conclusions can be drawn from these results. First that all the D type strains immunize mice readily with no significant differences in effectiveness from second to tenth passages. The O strains are all less effective than the D strains but there is an apparently significant progressive development of immunizing power with passage in the O state. The difference is particularly well shown between second and tenth passage strains, the fifth and eighth having too low a titre to give satisfactory results.

TABLE 14.

Immunization of mice with O and D type virus.

Number and type of virus fluid.			F/G ratio.	Titration *in ovo* Amniotic.	Allantoic.	Immunizing dose. O	D
67809	2nd	O	30/320	7·0	4·8	6·0	
67815	2nd	D	320/360		8·2		3·8
65717	5th	O	5/20	6·3	4·0	5·0	
65770	5th	D	40/40	7·0	7·5		4·0
66187	8th	O	25/120	5·5	3·7	4·8	
66480	8th	D	300/200	8·0	8·5		3·5
69350	10th	O	35/200	6·7	3·8	4·4	
69531	10th	D	480/240		7·5		3·1
69534	10th	D*	160/80		7·8		3·2

F/G ratio = Hirst agglutination titre fowl cells/guinea-pig cells.

Titration results are shown as the log. of 50 p.c. infective dose and immunizing dose as the log. of the number of doses needed to reduce the average lesion to grade 2 of partial consolidation.

Differential action of human tears on O and D strains. In this laboratory Beveridge has shown that human tears have a high power of inhibiting agglutination of red cells by the usual stock influenza viruses. If, for instance, falling dilutions of pooled human tears are mixed with a standard dilution of "Melbourne" allantoic fluid virus, the mixtures left overnight in a refrigerator and fowl cells added next morning, the endpoint of inhibition is at 1: 1,280 or 1: 2,560. With the same technique, except that human red cells were used, two stocks of BEL O gave endpoints of 15 and 10 respectively. These stocks were in the form of glycerolated amniotic fluid, so parallel experiments were made with BEL D in similar form and with human cells as indicator of inhibition. An endpoint of 180 was obtained in the same range as the values obtained when normal material was tested with fowl red cells (250 and 120). There is a very sharp difference in behaviour therefore between O and D strains of BEL. It is hoped to continue the investigation of this inhibition by tears along several lines but in the present paper we wish only to record the facts which indicate another distinction between the O and D phases.

DISCUSSION.

This work on the changes which take place immediately after isolation of influenza virus strains from human infections finds its justification at the present time in the possibility of its application to the problem of immunization against influenza. For the past three years we have been actively engaged in attempting human immunization by the nasal administration of living attenuated influenza

virus strains. Most of the work is still unpublished but the experience has been sufficient to suggest that the method can be safely used on a large scale and that in a considerable proportion of treated individuals there is a rise in antibody and a resistance to reinfection with the same attenuated strains some months later. The greatest difficulty in the work has been to know at what stage an influenza virus strain becomes of sufficiently reduced virulence to be safely used for human immunization. In our earlier work we had hoped that the virulence of a strain for ferrets might be an indication of its virulence for non-immune human beings. Our experience however indicates that A type strains which have lost their power to produce symptoms in ferrets, e.g. the fully adapted "Melbourne-Egg" chorioallantoic strain, produce as a rule neither symptoms nor antibody response in human beings. The observation of the sharp changes in F/G ratio associated with the early stages of chick embryo passage of the strains isolated in 1942 seemed to offer a new lead toward a method of estimating human virulence without the necessity for direct test in susceptible subjects.

The more immediate aim of the work was to gain some understanding of the nature of the primary change from what we have called the "original" O type to the "derivative" D type and to assess its significance in regard to epidemic influenza. The most recent comprehensive discussion of variation in viruses is that of Findlay (1940). In this review Findlay points out the so-far unresolved difficulty of determining whether the numerous and practically important examples of virus variation represent "dauermodificationen" resulting from the direct influence of growth and replication in an abnormal host or genetic mutations selectively favoured by the new environments in which the changes appear. As far as we are aware the method used in the present work of passage at limiting dilutions has not previously been applied to the virus problem. The development of egg culture methods and the various applications of the Hirst agglutination test have made it technically practicable to apply essentially the same methods to the problem of influenza virus variation as one would apply to a similar problem in bacterial variation. For practical purposes the amniotic or allantoic cavities of the chick embryo can be considered analogous to tubes of nutrient broth and the changes under consideration are not essentially unlike say the appearance of lactose-fermenting variants from a strain of "B. coli mutabile" grown in lactose broth. In the absence of plating methods a fairly clear idea of the process of mutation and of overgrowth by the lactose-fermenting variant could be obtained by subinoculation of serial dilutions into further lactose tubes with observations of pH changes taking the place of the F/G ratio determinations in the virus problem.

Our results have established the existence of two forms or phases of influenza virus A—"O" and "D"—most easily distinguished in practice by the differential Hirst test and by the fact that while both phases will infect amniotically only the D form will multiply after allantoic inoculation. Only on one occasion have we obtained any evidence of the existence of the D phase in a human throat filtrate and we consider it highly probable that only the O phase is concerned in a typical human epidemic such as the one we studied in May, 1942.

When filtered human material containing only O type virus is inoculated amniotically certain of the cells in contact with amniotic fluid are infected and increasing amounts of virus are set free into the amniotic fluid. After two or three days incubation this fluid contains, in addition to phase O virus, a variable but usually smaller amount of phase D virus. To account for its appearance alongside but not completely replacing O, we have no alternative but to postulate the occurrence of a discontinuous mutation. The change is clearly not a necessary result of a certain period of time spent or a certain number of replications made in the embryo tissues. We have O strains which have spent eleven or twelve passages (more than thirty days) in chick tissues and D strains with only two passages.

From a consideration of the results of comparative amniotic and allantoic titrations it seems probable that if even one infective dose of the D phase is inoculated amniotically it will have overgrown the O phase (assuming this to have initially been present in excess) within three days. All the results are compatible with the hypothesis that mutation from O to D takes place with some definite frequency, perhaps once in every 10^5 or 10^6 replications of virus type O and we have direct evidence that the D type multiplies more rapidly than the O. The results of inoculating pure O material will depend therefore mainly on the time elapsing before the first mutation to D occurs. If, as we must assume, the occurrence of mutation is governed statistically by some uncontrolled intrinsic or extrinsic (e.g. penetrating radiation) factors, the change to D will occasionally occur much earlier in some embryos than in others inoculated with the same dilution. This will account for the findings shown in Fig. 2 where it will be noted that an occasional D type fluid appears amongst the predominant O type fluids of high dilutions but we have never observed a discrepant O fluid appearing amongst the D fluids of the lower dilutions.

The fact that one primary filtrate from an influenza patient ROW produced allantoic fluid infection in two of four embryos was recorded in a previous paper (Burnet *et al.*, 1942). The significance of this finding was not appreciated at the time and none of the primary filtrate was preserved for further investigation. It does however suggest the importance of determining to what extent the O to D change can occur in the human subject. The methods developed in the present work can be readily applied to the analysis of filtrates from both human and ferret infections. Until this is done we can only speculate on whether the O to D change occurs in human infections. Our general impression from the whole investigation is that human influenza virus A in its typical O phase is genetically relatively unstable. The more stable D phase will appear whenever the virus has the opportunity to multiply sufficiently to provide an adequate number of replications. In the common laboratory hosts, mouse and chick embryo, the D type multiplies more freely and overgrows the O type. The well-known fact that as a rule two or more ferret passages are necessary before a newly isolated influenza strain can be adapted to mouse passage suggests that the same conditions hold in ferrets. If this view is correct one must ascribe to the O form some specific character favouring its survival under the conditions of a human epidemic. The fact that human tears have far less inhibitory action against the O type than against stock D type strains provides a possible lead as to the nature of those specific characteristics.

Finally we should like to draw attention to the curious parallelism between susceptibility of red cells to agglutination by O type virus and susceptibility of the corresponding species to infection with that type. Human and guinea-pig cells are highly susceptible, ferret cells are less susceptible but are as readily agglutinated by O as by D type strains. All the other species tested notably fowl and mouse are much less readily agglutinated by O than by D strains. Only human beings and ferrets are clinically susceptible to influenza but it has been shown above that the guinea-pig responds to O type inoculation intranasally by a sharp antibody response. We have no hamsters available but judging from the work of others (Taylor and Parodi, 1942) they are also susceptible to O type virus. We should predict with some confidence that their red cells will be found to resemble those of man and guinea-pig in being susceptible to O type virus.

SUMMARY.

Strains of influenza virus A isolated by amniotic inoculation in the Victorian epidemic of May, 1942, have shown a uniform change in character on passage in chick embryos.

This change described as from the O (original) to the D (derivative) form has been studied in detail for one strain, BEL.

The O form (e.g. that found in the amniotic fluid of embryos inoculated with throat filtrates from human patients) has the following characteristics:

1. Haem-agglutination tests show a much higher titre with guinea-pig red cells than with fowl cells: the ratio F/G ranges from 0·05 to 0·2.
2. The virus fails to multiply when inoculated into the allantoic cavity of twelve day chick embryos.
3. There is, on the average, an increase in the amount of amniotic fluid in embryos infected with O type virus.
4. The virus is not inhibited in haem-agglutination tests by human tears.

The D form differs in the following respects:

1. Haem-agglutination tests show an F/G ratio near unity (0·5 to 2·0).
2. The virus multiplies readily in the allantoic cavity.
3. On amniotic infection the average amount of amniotic fluid is reduced.
4. It is readily inhibited in haem-agglutination tests by human tears.

On "titration-passage" of O strains by the amniotic route lower dilutions give D type fluids while the higher dilutions give as a rule O type fluids. From a quantitative consideration of the results of such passages it is concluded that the O → D change is a discontinuous mutation.

D type virus infects mice more readily and multiplies more rapidly in the chick embryo (amniotic route) than O type. The O type is fully virulent for ferrets and infects guinea-pigs intranasally somewhat more actively than the D type.

Slight serological differences between the two types can be detected using ferret immune sera.

The possible significance of the O → D change in human infections is discussed.

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TITRATION OF ANTIBODY AGAINST INFLUENZA VIRUSES BY ALLANTOIC INOCULATION OF THE DEVELOPING CHICK EMBRYO

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GENERAL CHARACTERISTICS OF THE METHOD.

The use of the amniotic and allantoic fluids as sources of high titre influenza virus was first described in 1940 (Burnet). The chick embryos had been inoculated by the amniotic method but subsequent work showed that a much simpler method could be used namely to inoculate into the allantoic cavity by simply inserting a needle through a small drill hole in the shell (Burnet, 1941; Nigg *et al.*, 1941; Henle and Chambers, 1941). The method has now become almost the universal procedure for producing virus for vaccine, for complement fixation or for general experimental work, but it has not been widely used as an experimental method in itself. The purpose of this paper is to describe the application of the method to the titration of virus and of antibody. In our opinion it is quite as satisfactory as mouse inoculation methods and if a regular supply of fertile eggs is maintained is much cheaper. Chorioallantoic methods are strictly limited to the very few suitably adapted strains and have never been used extensively in influenza virus work outside of this laboratory. The allantoic method is applicable to all strains except those which have been maintained in the completely unadapted "human" state.

The principle of the method is to inoculate eggs with the dilutions or mixtures being studied and after three days' incubation to test a few drops of the allantoic fluid for its power to agglutinate fowl red cells (Hirst, 1941). A sharply distinct positive or negative result is obtained from every surviving embryo.

Details of Technique.

Eggs after 12 days' incubation are normally used but 10 or 11 day eggs are equally satisfactory. The eggs are candled and a small pencil mark made over a region where the membrane is well developed but away from any large chorioallantoic blood vessel. A short channel is cut with the drill over the region marked so that a fine hypodermic needle can be easily pushed into the allantoic cavity.

The shell in the region of the opening is lightly brushed with sterile melted paraffin wax to avoid any contamination of fluid which may ooze out and then be drawn in again during the process of inoculation. The eggs are inoculated with 0·05 ml. of fluid with a tuberculin syringe and fine needle and the puncture sealed with a drop of melted paraffin. The eggs are then incubated at 35° C. for three days.

More recently we have adopted an alternative method of allantoic inoculation which is more convenient for anyone accustomed to using a pipette with teat. The eggs are prepared as before and are arranged on a tray so that the paraffin-coated area around the drill hole is approximately horizontal. If a drop of fluid is placed on the wax and then the allantoic cavity is punctured by thrusting a dissecting needle through the fluid and the waxed shell, when the needle is withdrawn the fluid flows into the allantoic cavity. Alternatively the puncture hole may be made first and then the fluid placed over it. In this way up to 5 ml. of fluid may be made to flow into the allantoic cavity. In practice the most convenient technique is to insert the needle and while it is

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still in position to bring the point of the graduated capillary pipette containing 0·5 ml. of inoculum to the site of insertion. Delivery of the inoculum is commenced before and completed after withdrawal of the needle. By this simple technique eggs can be inoculated very rapidly and with certainty that all the inoculum has entered the allantoic cavity.

After incubation the eggs are opened and some of the allantoic fluid taken for testing. Sufficient fluid can be withdrawn with the egg lying on its side on a tray by puncturing the site of the original inoculation with one point of a pair of sharp pointed forceps and inserting a rather fine capillary pipette. (If the eggs are again sealed and incubated many will hatch.) Dead embryos can be recognized by pink discolouration of the fluid. Negative fluids from dead embryos are of no significance as the death may be non-specific and have occurred before there was time for virus multiplication.

The usual procedure is to have a rack of tubes each containing 0·25 ml. of saline. As each egg is opened 3 or 4 drops of allantoic fluid are placed in one of these tubes and when the series is completed 0·25 ml. of fowl red cells (2 p.c. suspension in saline) is added to each tube. Agglutination is read after an hour on the bench. The results are recorded as + or — and the end points calculated according to Muench's method as described by Parker and Rivers (1936).

In practice it is found that with all normal laboratory strains the results can be read almost as well from the appearance of the fluids as from the Hirst agglutinations. Fluids from negative embryos are either perfectly clear or turbid with urates, positive fluids practically never show urate deposits and have a light turbidity due to cellular debris which has a very different appearance from the opalescence of commencing urate deposition. Occasionally however eggs inoculated with limiting dilutions show macroscopically normal fluids which give a positive Hirst test.

Direct Titrations.

As an example of the results obtained in direct titration of virus the experiment recorded in Table 1 may be cited. The virus used was part of a batch of attenuated "W.S." virus prepared for intranasal immunization. Tenfold dilutions were prepared using in this as in all other experiments ice cold saline containing 10 p.c. of normal horse serum as diluent. Nine eggs were inoculated for each dilution.

TABLE 1.

Titration of a typical allantoic fluid virus (W.S.).

Dilution.	Results. Living embryos.	Dead.	Reed and Muench Accumulated totals. +	—
10^{-10}	— — — — — — — —	—	0	25
10^{-9}	— — — — — — — —	—	0	17
10^{-8}	+ + — — — — — —	— —	2	9
10^{-7}	+ + + + — — — —	—	6	4
10^{-6}	+ + + + + + + +	+ + —	12	0

Only surviving embryos are used in determining the titre which in this instance is $10^{7·2}$ or since 0·05 ml. only is used for inoculation the titre can be expressed as $10^{8·5}$ (50 p.c.) infective doses per ml.

Antibody Titrations.

Antibody titrations are made along orthodox lines. The virus used is in the form of pooled allantoic fluid from several embryos inoculated at the 10th day of incubation. The eggs are chilled after 48 hours incubation at 35° C. and the whole of the allantoic fluid collected. The fluid is clarified by light centrifugation or, preferably, by filtration through a sintered glass filter. Serial tenfold dilutions of virus are prepared in cold horse serum saline and if necessary five- or ten-fold dilutions of serum in the same diluent. Mixtures of equal volumes of virus and serum suitably diluted are held in the refrigerator for about an hour and then inoculated in 0·05 ml. volume into 12 day embryos, either three or four eggs being used for each mixture.

In Table 2 are shown results obtained with a strain of virus BEL isolated in the May, 1942, epidemic, an account of which has recently been published (Burnet *et al.*, 1942). Titrations of the homologous ferret serum F174 and of acute and convalescent serum from two patients CON and MOO are included.

At the foot of the Table the antibody titres are expressed (A) in terms of the activity of the homologous ferret serum taken as standard. Since this method gives inconveniently low values for weak human antisera we have generally adopted method (B) in which the titre is expressed as the log. of the number of infective doses neutralized by undiluted serum. Thus from the data in Table 2 the 50 p.c. end point of the direct virus titration is $10^{7·7}$ or allowing for the 1: 2 dilution of virus in serum mixtures $10^{7·4}$. With F174 serum the end point is $10^{-0·3}$ and the log of the number of infective doses neutralized 7·7.

TABLE 2.

Antibody titrations by allantoic inoculation.

A. *Preliminary titration of virus stock BEL.*

10^{-6}	+ − − −	
10^{-7}	+ + + +	Titre 7·7
10^{-8}	+ + + +	

B. *Antibody titrations against virus BEL.*

	Serum				
Mixtures.	F174.	CON I.	CON II.	MOO I.	MOO II.
S + V 10^{-5}		+ + −		+ + +	
S + V 10^{-4}		+ + +		+ + +	
S + V 10^{-3}		+ + +		+ + +	
S + V 10^{-2}					
S + V 10^{-1}	− − − −		− − −		− − −
S + V	− − − −		+ − −		− − −
S/5 V	+ + + +		+ + +		+ − −
S/25 + V	+ + + +		+ + +		+ + +
Titre A	1	0·000002	0·5	<0·000002	5
Titre B	7·7	2·2±	7·5	<2·2	8·2

S = undiluted serum, V = undiluted virus. S + V 10^{-5} = mixture containing equal volumes of undiluted serum and virus diluted 1 in 10^5.

APPLICATIONS OF THE ALLANTOIC TITRATION METHOD.

The Response of Human Beings to Natural Infection.

Acute and convalescent sera from influenza patients in the May, 1942, epidemic in Victorian Military Camps were available. The results of titrating these sera against BEL are shown in Table 3. It will be seen that the 19 subjects tested fall rather sharply into three groups: I, 10 show practically no initial antibody and an enormous subsequent rise; II, 8 show equally low

TABLE 3.

Titration of acute and convalescent sera from influenza patients.

Patient.	Hirst titration.	Allantoic titration. Titre of 1st serum.	2nd serum.	Antibody rise.
CON	15–240	2·3±	7·5	200000 ×
MOO	30–960	<2	8·2	>2000000 ×
BEL*	30–640	<2	8	>1000000 ×
ROW*	15–240	<2	7·2	>100000 ×
QUI	25–240	2	8·2	>1000000 ×
HOB	30–320	<2	7·8	>1000000 ×
VAU	30–200	1	8	10000000 ×
JUB	20–320	3	8	100000 ×
YAX	50– 80	<2	6·5	>300000 ×
KIT	20–120	<1	4	>1000 ×
BIS	20–160	Nil	Nil	Nil
TUR	20– 80	<1	1·5	10 × (±)
BAN	10–120	2	3	10 ×
DOW*	30– 60	<2	<2	Nil (?)
McC	60–120	Nil	Nil	Nil
GRA*	30–120	<1	2·5	100 × (±)
YOU	10– 80	<2	2	10 × (±)
SPI	20– 80	1·5	1·5	Nil
FOX	65–240	6·2	8	100 ×

Hirst titres obtained as described by Burnet *et al.* (1942).
Allantoic titres $\log_{10}$ number of 50 p.c. infective doses neutralized by undiluted sera.
Nil = no neutralizing activity detected. * Patients from whom virus was isolated.

initial antibody and show only a trivial rise not exceeding 100 fold; III, one subject only showed a relatively high initial level with a subsequent rise to the same average level as group I. It will be seen that there is a fairly good correlation with Hirst antibody titres. The mean values are shown for convenience in Table 4. The most striking feature of the Table is naturally the extremely wide range of antibody titres by the allantoic method. Where there is a large Hirst rise averaging about 12 fold the allantoic rise is around a million fold but in the group with little or no allantoic rise the Hirst titre increases only about four fold. Similarly if we take the initial titres all the subjects in groups I and II have a low Hirst titre around 25 and practically no allantoic antibody while the one subject in group III shows values respectively 65 and 6·2.

TABLE 4.

Correlation of Hirst antibody with allantoic antibody changes.

	Number of subjects.	Average Hirst antibody. 1st bleeding.	2nd bleeding.	Mean allantoic antibody. 1st bleeding.	2nd bleeding.
Group I	10	26·5	336	0–2	7·5
II	8	25	102	0–2	0–3
III	1	65	240	6·2	8

Despite the virtual absence of allantoic antibody rise the sera falling in group II show definite and sometimes large antibody increases when titrated by standard methods in mice. Five of the pairs were tested against the strain "Melbourne" in mice with the results shown in Table 5.

TABLE 5.

Mouse titrations of antibody in sera of group II patients.

Patient.	1st Serum.	2nd Serum.	Antibody rise.
BIS	S + V/ 3	S/ 5 + V	15 ×
BAN	S + V/ 20	S/10 + V	200 ×
DOW	S + V/ 10	S/ 2 + V	20 ×
McC	S + V/100	S + V	100 ×
GRA	S + V/200	S/ 5 + V	1,000 ×

Antibody titres shown in form of the serum (S) — virus (V) mixtures giving average grade 2 lesions in inoculated mice. Virus = supernatant fluid from 10 p.c. suspension of mouse lung in broth.

Comparison of Initial Antibody in Influenza Patients and in Unselected Subjects.

In the previous section it has been shown that of 19 typical influenza cases only one showed antibody detectable by the allantoic titration method against a homologous strain at the time of the illness. This suggested the possibility that the allantoic-antibody level might be a useful index of susceptibility to influenza. For comparison with these patients, all of them soldiers in camp, we had available a group of sera from about 100 soldiers who volunteered in February, 1942, for a test immunization with mixed attenuated virus. These sera had been stored for some months but any change resulting from storage would be to reduce the apparent antibody titre. A group of 36 of these sera taken before immunization was tested primarily with the single dilution of virus BEL 10^{-6}. Where 3 or 4 of the 4 eggs inoculated were infected at this dilution the serum was given a value of < 2 and regarded as showing no antibody. Most of the sera showing neutralization at this dilution were further tested to determine their approximate titre. The results are tabulated against the Hirst antibody titres (determined against strain "Melbourne") in Table 6.

TABLE 6.

Comparative Hirst and allantoic antibody titres of 36 normal subjects.

Hirst titre.	Allantoic titres.	Proportion with significant antibody.
10	0* 0 0	0/ 3
10– 18	0* 0 0 0 0 0 0 0 0 2, 3–4, 5, 5, 6	4/14
20– 35	0* 0* 0* 0 2, 2, 2·5, 5	2/ 8
40– 70	0, 2·5*	1/ 2
80–150	0, 2, 3, 6, 7·5	3/ 5
160 +	0, 3, 6·5, 6·5	3/ 4

* These subjects showed an antibody rise by Hirst tests against "Melbourne" after intranasal inoculation with mixed attenuated virus. 0 is equivalent to < 2.

If we compare the two types of antibody titration in the two groups it seems likely that in May, 1942, it was particularly those subjects with low Hirst titres and no significant allantoic antibody who contracted clinical influenza. The Hirst titres in the two groups are not absolutely comparable as strain "Melbourne" was used for the normal series but any discrepancy would probably be small. In 36 normal subjects there are 13 with detectable allantoic antibody, 9 giving a value of 3 or more, while in 19 influenza patients there are 2, only one having antibody over 3.

Changes in "Swine" Antibody Associated with Influenza A.

In order to establish the behaviour of swine type antibody estimated by the allantoic method several pairs of sera from the May, 1942, epidemic were tested against an allantoic fluid strain of Shope's "Swine 15" influenza virus. (Table 7.)

TABLE 7.

Comparative antibody titrations against swine influenza with acute and convalescent sera.

		Allantoic test.			Mouse test.		
Patient.	Hirst titres.	1st	2nd	Rise	1st	2nd	Rise
CON	< 10– < 10	< 2	? (see text)	?	Nil	S + V/5	100 ×
HOB	20– 120	4·5	7·3	600 ×	S + V/5	S/2 + V	10 ×
LYE	< 10– < 10	4·1	4·5	? 2 ×	Nil	Nil	Nil
MOO	30– 480	< 3	8·4	> 100,000 ×			
BEL	< 10– 40	3·7	8·1	25,000 ×			

Swine virus 50 p.c. end point $10^{7\cdot7}$ (7·4). Immune ferret serum F176 (homologous) 7·8.
Titres expressed as in Tables 3 and 5.

These results show an interesting lack of agreement amongst themselves. Two patients MOO and BEL both showing a very active homologous antibody rise, show a rise in "allantoic" Swine antibody of from 10,000 to 100,000 fold from an initial low level. HOB shows a moderate initial antibody level and a well marked rise by all three methods of titration. CON shows no initial antibody whatever by any method; mouse antibody rises sharply, Hirst not at all, while the allantoic results for the second serum gave no endpoint, positive and negative fluids being obtained from all the mixtures tested. Finally LYE shows no antibody by Hirst or mouse tests either before or after infection, while by allantoic titration a moderate level is present at both bleedings, essentially unchanged as a result of the influenzal attack.

Specificity of Antibody Response in Regard to Different A Subtypes of Influenza Virus

It is generally accepted that adults convalescent from an attack of influenza A show a relatively nonspecific antibody response which by either mouse (Horsfall and Richard, 1941) or chorioallantoic titration methods (Burnet and Lush, 1938) is equivalent against both "Melbourne" (corresponding to P.R. 8 of American or the "intermediate" group of British authors) and "W.S." subtypes. The rise against "Swine 15" is also nearly equivalent by the mouse titration method. In children the response is specific for the subtype involved in the infection as far as can be judged from the limited data presented by Burnet and Lush (1938).

TABLE 8.

Titration of human sera against W.S. by allantoic method.

	Titre against W.S.		Titre against BEL.	
Patient.	1st bleeding.	2nd bleeding.	1st bleeding.	2nd bleeding.
CON	2	2	2·3	7·5
QUI	2	2	2	8·2
HOB	2	2	2	7·8 (7·2)
VAU	N.T.	2·2	1	8 (7·7)
Ferret 166	N.T.	7·6		
Ferret Pool	N.T.	6·3		

Titres against BEL taken from Table 3 except for those in brackets which were done in parallel with the W.S. titrations. Both ferret sera were from animals experimentally infected with W.S. N.T. = not tested.

By the allantoic titration method the response in four patients tested from the Victorian 1942 epidemic is specific for the homologous type, no rise being shown against W.S. The results are shown in Table 8. The W.S. strain had been derived from the standard mouse passage strain

by 100 amniotic passages and was used in the form of allantoic fluid. Appropriate control tests were done to show (1) that the virus was neutralized typically by two W.S.-immune ferret sera, and (2) that the convalescent sera still showed typical activity against the homologous strain BEL.

DISCUSSION.

The results described show that the allantoic method can be satisfactorily applied to the titration of antibody in human or ferret immune sera against influenza virus A ("Melbourne", "W.S." and "Swine" subtypes). The results reported in the paper by Bull and Burnet (1943) show that the method can also be used to measure the antibody response against influenza B in immunized subjects.

There is every reason to believe that it would be equally applicable to studies involving any virus capable of multiplying freely in the allantoic cavity and producing fluid with active haem-agglutinating powers. This is perhaps the greatest advantage of the method since it can be used for any type of influenza virus except completely unadapted human virus (Burnet, 1942) without the necessity for special adaptation to mouse or chorioallantoic passage.

There have been at least five different methods described for estimating the change in antibody associated with convalescence from influenza. These are: (1) protection of ferrets from intranasal infection, (2) intranasal titration in mice, (3) complement fixation with several types of antigen, (4) titration on the chorioallantois against egg adapted strains, (5) Hirst's method using fowl cell agglutination. The present allantoic method makes a sixth.

No worker has studied all six reactions in parallel but from a survey of the literature and from our own experience it can be said that no two tests give fully concordant results. If one takes the most commonly used test (mouse lung titration) as standard it can be stated that complement fixation results show no systematic relationship to this, that the Hirst test titres resemble the mouse ones but are compressed in the sense that a hundred fold rise in mouse titre is likely to show a four to eight fold rise by Hirst's method, that there is a general parallelism between mouse and chorioallantoic titres but wide discrepancies may occur under special circumstances since the chorioallantoic seems to depend mainly on a relatively small proportion of high grade antibody.

If antibody titration is to have any more practical significance than to serve merely as an index to the fact of infection with the virus, it is important to find out which if any type of antibody is quantitatively correlated with resistance to natural influenza. The only justification for describing yet another antibody titration method is that our results suggest that the allantoic method may have this desirable feature. Of 19 persons who proved their susceptibility by becoming naturally infected, only 2 had any allantoic antibody and only one any significant amount, while in 36 unselected individuals of similar age and environment the corresponding numbers were 13 and 9. Further, within this group, 6 persons were proved susceptible to infection by the corresponding attenuated vaccine strain. One of these had a trace of allantoic antibody, the other five none.

The rather sharp division of patients with proved influenza into those showing a great rise of allantoic antibody and those showing little or none cannot be interpreted without much more investigation but it at least presents for consideration the hypothesis that the first group derive an effective immunity as a result of their attack while the second do not.

The potential importance of the allantoic titration method lies in the promise that it may give a more definite measure of resistance to influenza than the current mouse or red cell agglutination tests. If this can be established it may be possible to develop immunization methods with more certainty and speed by using the allantoic antibody response as a presumptive index of their efficiency.

SUMMARY.

By using the Hirst agglutination reaction as an indicator for virus multiplication, allantoic inoculation of chick embryos can be used as a cheap, simple and reliable method for titration of influenza viruses and the corresponding antibodies.

Using this method for the examination of 19 pairs of acute and convalescent sera from influenza A patients, the patients could be divided into two main groups, those showing a very large antibody increase and those showing little or none.

Initial bleedings from 19 influenza patients showed 18 with very low antibody while in 36 comparable unselected persons 9 showed moderate to high amounts of antibody.

Applications of the method to the titration of "Swine" influenza antibodies and to the rise of influenza B antibody after intranasal inoculation are given.

Following influenza A, the antibody rise shown against the homologous subtype is not evident when the tests are made against another A subtype (W.S.).

It is possible that antibody measured by this method gives the best indication of resistance to infection that has yet been developed.

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Since this paper was submitted for publication we have received Hirst's paper (J. Immunol., 45, p. 285, 1942) in which he describes an essentially identical method of influenza virus titration.

STUDIES ON THE *CLOSTRIDIUM OEDEMATIENS* GROUP

1. "H" AND "O" ANTIGENIC ANALYSIS

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INTRODUCTION.

In 1894 Novy isolated from guinea-pigs which died after injection of a contaminated solution of casein the organism since known as *Clostridium novyi*. Weinberg and Séguin (1915) later rediscovered it from cases of gas gangrene in soldiers under the name *B. oedematiens*. Since then several related species of clostridia have been isolated from diseases of animals, and bacteriologists now recognize them as members of the "*oedematiens*" or "*novyi*" group. To emphasize this Scott, Turner and Vawter (1934) suggested that the following "types" of the species *Cl. oedematiens* be recognized:

Type A: "classical" type of Weinberg and Séguin, *Cl. novyi* of American authors or *Novyscher Bazillus des malignen Oedems* of German authors. Found in from 16 to 48 p.c. of cases of human gas gangrene during the 1914–1918 World War (Zeissler and Rassfeld, 1928). Frequently found in wound infections in many species of animals.

Type B: the larger, non-glycerin-fermenting black-disease type and closely related *B. gigas* isolated by Zeissler and Rassfeld (1929) from cases of bradsot of sheep in Germany. *B. gigas* isolated on four occasions in Germany from cases of "sudden death" in man (Ziessler, 1934; Zeissler and Stoeckenius, 1937).

Type C: the bacillus of *osteomyelitis bacillosa bubalorum* ("O.B.B") isolated by Kraneveld (1930) from cases of bacillary osteomyelitis of water buffaloes in the Dutch East Indies. Regarded by Zeissler and Kraneveld (1929) as a non-pathogenic variety of *B. gigas*.

Cross-protection tests reveal no differences between the classical *Cl. oedematiens* and the black-disease type (Turner and Davesne, 1928; Turner, 1930), between *Cl. oedematiens* and *B. gigas* (Miessner, Meyn and Schoop, 1931), or between the black-disease type and *B. gigas* (Zeissler, 1931; Turner and Eales, unpublished).

Weinberg, Nativelle and Prévot (1937) included in the *oedematiens* group *Cl. haemolyticum*, described by Vawter and Records (1926) as the cause of bacillary ictero-haemoglobinuria of cattle in Nevada, U.S.A.. This organism produces a very characteristic pathological effect in inoculated guinea-pigs, including haemorrhagic oedema and intense icterus, and thus differs from *Cl. oedematiens*; furthermore, there is no cross protection between it and the classical *Cl. oedematiens* (Vawter and Records, 1926), nor between it and the black-disease type (Turner, 1930). Nevertheless, the investigations of Demnitz (1934) and of Sordelli and Ferrari (1937) show that certain toxic components may be shared by *Cl. oedematiens, Cl. haemolyticum* and *B. gigas* (bradsot type).

Before the relationships between these organisms can be accurately defined and a rational classification devised, more information including adequate knowledge of their serological relationships is necessary. With the exception of a study by Kreuzer (1939) of single strains of *Cl. oedematiens, Cl. haemolyticum* and *B. gigas* (bradsot type) nothing has been published on this subject. We, therefore, carried out "H" and "O" antigenic analysis of a representative collection of 33 strains, comprising 5 classed as *Cl. oedematiens* type A in the Scott-Turner-Vawter (S.T.V.) classification, 22 classed as type B and 6 classed as type C, as well as 2 strains of *Cl. haemolyticum*. For interest we included one strain of *Cl. oedema-*

toides not regarded as having any close affinities with this group. The strains used in this study are enumerated and some of their particulars are given in Table 1.

TABLE 1.

Particulars of strains studied.

Species.	Strains.	Isolated from Host.	Isolated from Disease.	Country of origin.	S.T.V. classification.
Cl. oedematiens (classical type)	Jolly, Weinberg	Man	Wound infection	France	Type A
	Hall 40	Horse	„	U.SA.	„ „
	Zeissler	Man	„	Germany	„ „
	Collins	Sheep	"Swelled Head"	South Australia	„ „
Cl. oedematiens (black-disease type)	Lister, Hopkirk	„	Black Disease	New Zealand	Type B
	Albiston, Tongala, B.D.10, B.D.19, B.D.24, B.D.58, LL.47, Mansfield, S.B.247N	„	„	Victoria	„ „
	R.B.977	Ox	„	„	„ „
	Rose 1, Rose 2, Rose 3	Sheep	„	New South Wales	„ „
	Oxer	„	„	Tasmania	„ „
	S.B.42	Horse	„	„	„ „
B. gigas (bradsot type)	6, 12A, 684, 1078, 1211	Sheep	Bradsot	Germany	„ „
B. gigas (O.B.B. type)	1, 2, 3, 4, 5, 6	Buffalo	Osteomyelitis of Buffaloes	Dutch East Indies	Type C
Cl. haemolyticum	H1 (Vawter and Records, 24407)	Ox	Bacillary icterohaemoglobinuria	U.S.A.	—
	T91* (= N.C.T.C. 3772)	„	„	Argentina	—
Cl. oedematoides	Hall 1303	Not known	—	—	—

* This is the strain referred to as *B. haemolyticus* "Hall" by Turner (1930).

TECHNIQUE.

All strains were selected after from 3 to 6 successive single-colony passages on blood agar plates incubated anaerobically by the method of Fortner (1928). Their cultural, biochemical and pathogenic relationships will form the subject of another communication.

With certain exceptions the serological technique in the main followed that of Henderson (1932). Suspensions for "H" and "O" analysis were made from 500 ml. amounts of 16- or 18-hour cultures in liver piece broth, which were filtered through gauze and cotton wool and then centrifuged. The deposits were washed twice in distilled water and resuspended in distilled water to give an opacity corresponding to 10 × No. 10 Brown standard (Burroughs and Wellcome).

Immunization of rabbits. "O" suspensions were prepared by heating the above suspensions at 100° C. for 2 hours, "HO" suspensions by incubating for 10 days at 37° C with 0·2 p.c. formalin to ensure detoxication. Both were made isotonic by adding 10 p.c. of an 8·5 p.c. solution of sodium chloride, thus reducing the opacity to 9 × No. 10 Brown standard. Before injection the formalin in the "H" suspensions was combined with ammonia. After collection of samples of pre-injectional sera, rabbits received courses of eight injections of "O" suspensions from 0·5 to 5·0 ml., or of six injections of "HO" suspensions from 0·5 to 3·0 ml., at intervals of two days; if necessary a series was repeated, until a satisfactory titre was obtained. Final bleedings were performed on the fifth and sixth days after the last injection, the samples bulked, filtered through Seitz E.K. pads, and stored at 4° C. without preservative. Pre-injectional serum samples contained no normal agglutinins in a dilution of 1 in 10. The sera for "H" agglutination were freed of "O" antibody by absorption with "O" suspensions.

Agglutination tests. Stock suspensions (10 × No. 10 Brown) prepared as above were used except that "H" suspensions were killed by heating at 60° C. for 1 hour. "O" agglutinations were carried out by the tube method. The suspensions were diluted to opacity No. 4 Brown, the saline content being adjusted to 0·42 p.c. and the total volume being 0·4 ml. The tests were carried

out at 45° C. and read after 4 hours and again after having stood at room temperature for 24 hours. For "H" agglutination, suspensions were generally too unstable for the tube test and hence a slide test was developed, the stock suspension being diluted to an opacity equivalent to 10 × No. 4 Brown standard and the saline adjusted to 0·42 p.c. The reactions were read to 10 minutes and were of the coarse floccular "H" type. Normal serum and suspension controls were included in all tests.

Agglutinin absorptions. For "O" absorptions, the packed centrifuged deposit from 1 volume of a 10 × No. 5 Brown "O" suspension was mixed with 1 volume of a 1 in 25 dilution of serum; the mixture was shaken and left at 37° C. for two hours and overnight at 4° C. After separation by centrifugation, the serum was tested in a final dilution of 1 in 100 for completeness of absorption. On some occasions absorption was repeated, but this was rarely necessary. "H" absorptions were carried out similarly using "HO" suspensions; one absorption was always sufficient.

"O" ANTIGENIC ANALYSIS.

Some difficulty was experienced with the "O" analysis through the necessity for repeated courses of injections with some strains.

With some strains we were unable to obtain "O" antisera with titres higher than 1 in 800; other strains gave better titres, as high as 1 in 25,600. The cross relationships were usually complete, i.e. there was significant cross agglutination in both directions; but in some cases it was partial, with significant cross agglutination in only one direction. The titres with heterologous strains were sometimes low and just within the range regarded by us as significant, i.e. 1 in 100. In some instances heterologous strains were agglutinated to much higher titres than the homologous. A similar phenomenon was observed by MacLellan (1939) with *Cl. tetani.*

Tabulated results of cross agglutinations between the 28 "O" antigens and the 27 "O" antisera are given in Table 2; they indicate a very close relationship between the 28 strains examined. The poor agglutination of some strains by the heterologous sera suggests that the members of the *oedematiens* group, although sharing at least one common "O" antigen, are antigenically complex.

The "T91" strain of *Cl haemolyticum* shares a common "O" antigen with classical *Cl. oedematiens,* its B.D. type, *B. gigas* (bradsot type) and *B. gigas* (O.B.B. type), but there is little "O" antigenic relationship between these organisms and the "H1" strains of *Cl. haemolyticum*; nevertheless, the two strains of *Cl. haemolyticum* apparently share a common "O" antigen.

There was no cross agglutination between *Cl. oedematoides* and any of the other strains.

In a preliminary attempt to analyse the distribution of "O" antigens, some "O" absorption tests were carried out, with the results shown in Table 3.

TABLE 3.

"O" agglutinin absorption tests.

	Reciprocal titre of absorbed serum against homologous strain.				
Absorbing strain.	"Jolly" (A).	"Lister" (B).	"BD24" (B).	"Gigas 12A" (B).	"O.B.B. 4" (C).
None	800	1,600	12,800	1,600	800
Jolly (A)	**0**	200	200	100	0
Lister (B)	100	**0**	100	0	0
Hall (A)	100	100	100	100	0
Zeissler (A)	200	100	200	100	0
Albiston (B)	200	100	100	0	100
Tongala (B)	200	100	100	100	100
Hopkirk (B)	200	100	100	0	0
Oxer (B)	200	100	100	Not done	0
BD10 (B)	200	100	400	100	100
BD19 (B)	400	200	0	0	100
BD24 (B)	200	200	**0**	0	100
BD58	100	200	0	0	0
Rose 3 (B)	200	100	100	0	100
Gigas 12A (B)	200	100	100	**0**	100
O.B.B. 4 (C)	200	100	200	100	**0**
Haem. H1	800	Not done	6,400	Not done	Not done
Haem. T91	400	400	800	0	200

O = no agglutination with a dilution of 1 in 100.

These absorption tests show that none of 14 strains representing the three S.T.V. types could completely absorb the agglutinins from serum against the type-A strain "Jolly", nor from the serum against the type-B strain "Lister". *Cl. haemolyticum* "H1" was unable to absorb agglutinins from "Jolly" antiserum, and *Cl. haemolyticum* "T91" only partly absorbed agglutinins from "Jolly" and from "Lister" antisera. On the other hand, the type-B strains "BD19" and "BD58" were able to absorb completely the agglutinins from antiserum against the type strain "BD24", whereas the other strains of the *Cl. oedematiens* group almost but not completely absorbed them. *Cl. haemolyticum* "H1" was almost without effect, whereas *Cl. haemolyticum* "T91" reduced the titre to one-sixteenth of the original. The type-B strains "Lister", "Albiston", "Hopkirk", "BD19", "BD24", "BD58", "Rose 3", and *Cl. haemolyticum* "T91" absorbed completely the agglutinins from serum against the type-B strain "Gigas 12A", and the other strains tested almost completely absorbed them. The type-A strains "Jolly" "Hall" and "Zeissler", and the type-B strains "Lister", "Hopkirk", "Oxer" and "BD58" completely absorbed the agglutinins from serum against the type-C strain "O.B.B. 4", and the other strains, including *Cl. haemolyticum* "T91", almost completely absorbed them.

The behaviour of the strains in the cross-agglutination and agglutinin-absorption tests can be explained reasonably well on the hypothesis that there are two *Cl. oedematiens* "O" antigens, common to all strains, which may be referred to as *Oed.* I and *Oed.* II, and that in some strains *Oed.* I is predominant and in others *Oed.* II. The two *Cl. haemolyticum* strains appear to possess two "O" antigens, which may be referred to as *Haem.* I and *Haem.* II, both represented in strain "H1" but with *Haem.* I predominant (or alone) in strain "T91"; in addition the two *Cl. haemolyticum* strains must possess a further component, either *Oed.* I or *Oed.* II, common to them and to *Cl. oedematiens*, but present in only relatively small amount in strain "H1".

With the data available a more detailed analysis cannot be attempted. More extensive absorption tests are required but unfortunately the investigation had to be concluded before this could be done.

Kreuzer (1939), after studying a single strain of *Cl. oedematiens* (i.e. Type A), *B. gigas* (i.e. Type B) and *Cl. haemolyticum*, concluded that *Cl. oedematiens* and *B. gigas* possessed common "O" antigens but that the "O" antigen of *Cl. haemolyticum* was quite distinct. This is compatible with our findings.

"H" ANTIGENIC ANALYSIS.

With the exception of *Cl. haemolyticum* "H1", the "H" sera were all of high titre, up to 25,600. The 27 sera were tested against the corresponding "HO" suspensions. In addition they were tested against 8 suspensions against which no antisera were prepared; these were type-B strains "Mansfield", "SB247N", "Gigas 684", 'Gigas 1,078" and "Gigas 1,211" and the type-C strains "OBB1", "OBB5" and "OBB6".

TABLE 4.

Summary of "H" antigenic analysis.

"H" Type.	Species.	Strains.	S.T.V. Type of *Cl. oedematiens*.
1	*Cl. oedematiens* (classical)	Jolly, Hall	A
2	"	Weinberg	"
3	"	Zeissler	"
4	"	Collins	"
5	*Cl. oedematiens* (B.D. type)	Lister	B
6	"	SB42	"
7	"	Rose 2	"
8	"	LL47	"
9	*Cl. oedematiens* (B.D. type)	BD10, RB977, SB247N	"
	B. gigas (bradsot)	12A	"
	Cl. haemolyticum	T91	—
10	*Cl. oedematiens* (B.D. type)	Albiston, Tongala, Hopkirk, Oxer, DB19, BD24, BD58, Rose 1, Rose 3, Mansfield	B
	B. gigas (bradsot)	6, 684, 1,078, 1,211	"
	B. gigas (O.B.B.)	1, 2, 3, 4, 5, 6	C
11	*Cl. haemolyticum*	H1	—

The results, which are summarized in Table 4, were quite clear-cut. There was no crossing between the "H' types with the following slight exceptions: "SB42" antigen, agglutinated by "SB42" antiserum in a 1: 12,800 dilution, was agglutinated 1: 400 by "BD24" antiserum, but "SB42" antiserum did not agglutinate "BD24" antigen; "Rose 2" antigen, agglutinated

1: 6,400 by its own antiserum, was agglutinated 1: 800 and 1: 100 by 'Hopkirk" and "BD24" antisera respectively, but "Hopkirk" and "BD24" antigens were not agglutinated by "Rose 2" antiserum. The 33 strains belonging to the *oedematiens* group of Weinberg, Nativelle and Prévot fell into 11 distinct "H" types, 7 of which contained only single strains. The strain of *Cl. oedematoides* did not react with any other strain and will not be further discussed.

TABLE 5.

"H"-agglutinin absorption tests.

"H" Serum.	Absorbed by suspension of	Tested against	Titre.	Remarks.
Jolly	Unabsorbed	Jolly	3,200	
		Hall	1,600	
Jolly	Hall	Jolly	0	Hall completely absorbs from Jolly and Hall antisera agglutinins against both Jolly and Hall.
		Hall	0	
Hall	Unabsorbed	Jolly	3,200	
		Hall	3,200	
Hall	Hall	Jolly	0	
		Hall	0	
BD24	Unabsorbed	BD24	6,400	
		Hopkirk	6,400	
		OBB2	6,400	
BD24	BD24	BD24	0	
		Hopkirk	0	Three selected members of this "H"-type (10) completely absorb agglutinins from antiserum prepared against one of them.
		OBB2	0	
BD24	Hopkirk	BD24	0	
		Hopkirk	0	
		OBB2	0	
BD24	OBB2	BD24	0	
		Hopkirk	0	
		OBB2	0	
RB977	Unabsorbed	BD10	1,600	
		RB977	6,400	
		Gigas 12A	12,800	
RB977	RB977	BD10	0	
		RB977	0	
		Gigas 12A	0	
RB977	BD10	BD10	0	
		RB977	0	Two strains of this "H"-type (9) completely absorb agglutinins from two antisera prepared against one of them and against a third strain.
		Gigas 12A	0	
Gigas 12A	Unabsorbed	BD10	1,600	
		RB977	1,600	
		Gigas 12A	3,200	
Gigas 12A	RB977	BD10	0	
		RB977	0	
		Gigas 12A	0	
Gigas 12A	BD10	BD10	0	
		RB977	0	
		Gigas 12A	0	

Of the 5 type-A strains, 4 were strain-specific, and strains "Jolly" and "Hall" fell into the one "H" type. Of the black-disease gigas group, i.e. type-B strains, one from a sheep in New Zealand ("Lister"), one from a horse in Tasmania ("SB24"), one from a sheep in New South Wales ("Rose 2"), one from a sheep in Victoria ("L.L.47"), and the "H1" strain of *Cl. haemolyticum* were strain-specific. Two strains from sheep in Victoria ("BD10' and "SB247N", one strain from an ox in Victoria ("RB977"), one strain from a sheep in Germany (Gigas 12A) and the "T91" strain of *Cl. haemolyticum* formed a small homogeneous "H" group. The remaining 20 strains, comprising 6 from sheep in Victoria ("Albiston", "Tongala", "BD19", "BD24",

"BD58", "Mansfield"), one from a sheep in New Zealand ("Hopkirk"), one from a sheep in Tasmania ("Oxer"), two from sheep in New South Wales ("Rose 1" and "Rose 3"), four from sheep in Germany ("Gigas 6", "684", "1,078" and "1,211"), together with the 6 strains of type C from buffaloes in Dutch East Indies (O.B.B. 1–6) fell into one large homogeneous "H" group.

Some "H" agglutinin absorption tests on three of these "H" types are summarized in Table 5. They demonstrate the complete "H"-antigenic identity of the type-A strains "Jolly" and "Hall", of the type-B and type-C strains "BD24", "Hopkirk" and "OBB2" and of the type-B strains "RB977", "BD10" and "Gigas 12A".

It appears likely that the classical *Cl. oedematiens*, i.e. type A, comprises a large number of quite separate "H" groups which do not share "H" antigens with each other or with any strains of the black-disease type of *Cl. oedematiens*, *B. gigas* (bradsot), *B. gigas* (O.B.B.) or *C. haemolyticum*. Some of the type-B strains (black-disease) also form separate "H" subtypes. On the other hand, it is likely that a large proportion of strains which would be classified as types B and C, comprising strains from a variety of hosts and of wide geographical distribution, would fall into one large homogeneous "H" type, and that a small proportion of type-B strains and some strains of *Cl. haemolyticum* (e.g. "T91") would fall into another "H" group. Some strains of *Cl. haemolyticum* (e.g. "H1") are quite distinct from the *Cl. oedematiens* group and from other strains of *Cl. haemolyticum*.

Kreuzer (1939) in his restricted study found that the single strain of *Cl. oedematiens* (i.e. type A), *B. gigas* (i.e. type B) and *Cl. haemolyticum* respectively had distinct "H" antigens. This is quite compatible with our findings

Precipitin Tests.

Interface precipitin tests were carried out by using as antigens acid extracts made by the method of Lancefield (1928) and alkaline extracts in 1 p.c. Na_2CO_3 similar to those used successfully in this laboratory by Rodwell (1941) in a similar study of the *Cl. welchii* group.

However the results with either sort of extracts were not very satisfactory with the *Cl. oedematiens* group. They confirmed the evidence of the "O" agglutination tests, that common antigenic components are shared by members of the group belonging to each of the three types and by the "T91" strain of *Cl. haemolyticum*, but they were difficult to analyse satisfactorily because some strains did not yield active extracts and some extracts reacted with heterologous sera but not with the homologous sera.

DISCUSSION.

The results clearly demonstrated a close "O" antigenic relationship between the various strains and species of the *oedematiens* group which were included in this study. Although evidence was obtained that at least two *oedematiens* "O" antigens were distributed among the group, the absorption tests were not sufficiently comprehensive to yield an accurate analysis of their distribution.

The "H" analysis emphasized the distinction between the "classical" gas oedema strains (S.T.V. type A) and the essentially "animal" strains and species (S.T.V. types B and C). However, even within these S.T.V. types many strains had no "H" antigenic relationships with any other strains. It was therefore surprising that a large proportion of strains of *Cl. oedematiens* (black-disease type), *B. gigas* (bradsot type) and of *B. gigas* (O.B.B. type), from a variety of animal hosts and from widely remote geographical sources, formed a homogeneous "H" group. It is apparent that a simple slide-agglutination test with an antiserum of this group would help in the identification of a large proportion of strains.

The relationship of the pathogenically distinct *Cl. haemolyticum* to the other members of the *oedematiens* group is not quite clear. Vawter and Records (1931) studied the cross agglutination reactions of formalin-killed suspensions of 16 strains of *Cl. haemolyticum*, isolated in Nevada, against sera prepared from living suspensions, the reactions thus being due to the "H" antigens; 15 strains formed a homogeneous group and there were no cross reactions with strains of *Cl. chauvoei*, *Cl. septicum* and *Cl. sordellii*. Instability of the one strain of *Cl. oedematiens* made it difficult to decide the relationship of *Cl. haemolyticum* to *Cl. oedematiens*. It is a matter of regret that in our study only two strains of *Cl. haemolyticum* were available. One strain, "T91", is serologically identical, so far as "H" antigens are concerned, with a small group of strains of the B.D. bacillus and of *B. gigas* and also shares common "O" antigens with all except one of the 33 strains studied, in-

cluding the type-A strains; the other strain, "H1", has distinct "H" antigens, but has a partial "O" relationship with 18 of the strains, including some type-A strains. More work is necessary before the relationship of *Cl. haemolyticum* to the *oedematiens* group can be clarified.

The existence of common "O" antigens among the various strains in this study supports the view of Scott, Turner and Vawter and of Weinberg, Nativelle and Prévot that they should be regarded as belonging to an *oedematiens* group; but the large number of "H"-types (at least 11) makes a serological classification of the group not only impracticable but of no useful relationship to what is known of the host-specificity, pathogenicity, or toxin-antitoxin neutralizations, since at least one "H"-type contains black-disease, bradsot and haemolyticum strains, and another contains black-disease, bradsot and the non-pathogenic O.B.B. strains. It appears that the S.T.V. type A contains at least 4 "H" (1–4) sub-types, the type B at least 4 "H" sub-types (5–8), that two other sub-types (9 and 10) contain type-B plus *Cl. haemolyticum* strains and type-B plus type-C strains, respectively, and that some strains of *Cl. haemolyticum* may have no "H" relationship to other strains in the group.

It is common practice to prepare vaccines against black disease by detoxicating whole cultures of toxic strains of *Cl. oedematiens* (black-disease strains) by means of formalin. It is believed that the efficacy of the vaccine depends upon the formalin toxoid; the bacterial cells are left mainly to avoid unnecessary expense of removing them. It is not known if the "O" antigens are important in active immunization against black disease, as Henderson (1932b) has shown for infection by *Cl. chauvoei*; but if it be so, the results of our "O" analysis indicate that it may be desirable to use strains which, besides being otherwise suitable, contain both the "O" antigens.

SUMMARY.

"H" and "O" antigenic analysis was carried out on 33 strains of clostridia belonging to the "*oedematiens*" group of Weinberg, Nativelle and Prévot; these comprised 5 strains of classical *Cl. oedematiens*, 17 strains of the black-disease type of *Cl. oedematiens*, 5 strains of the "*B. gigas*" from cases of bradsot, 6 strains of the non-pathogenic "*B. gigas*" isolated from osteomyelitis of buffaloes and 2 strains of *Cl. haemolyticum*.

Evidence was obtained that these strains share two "O" antigens in various proportions.

Many strains did not share "H" antigens with other strains, but 20 strains comprising black-disease, bradsot and osteomyelitis strains formed a homogeneous "H" type.

The evidence supports attempts to group the strains examined as members of an "*oedematiens*" group but gives little hope for a useful serological subdivision.

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STUDIES IN THE PHYSIOLOGY OF HOST-PARASITE RELATIONS[1]

4. SOME EFFECTS OF TOMATO SPOTTED WILT ON GROWTH

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The problem of virus diseases in plants is a complex one, the successful elucidation of which requires investigations starting from many angles. In the case of tomato spotted wilt (T.S.W.) attention has largely been concentrated on the determination of the properties of the virus (Samuel *et al.*, 1933, 1935; Best, 1936, 1939). The present work forms a contribution to another aspect of the T.S.W. problem—that of the relative growth and metabolism of healthy and infected plants—in an endeavour to obtain quantitative information on the methods of action of the virus.

The period of growth in the experiments to be described, covers the interval between the expansion of the second-third and the eighth-ninth leaves of seedling tomato plants. During this early part of the grand period of growth, the morphological development of the healthy plant is proceeding rapidly. Dry weight, which provides the most comprehensive summary of growth, together with height and leaf area are used as criteria of growth in relation to T.S.W. infection.

For purposes of comparison of the rate of increase in dry weight of healthy and of T.S.W. diseased plants during successive periods of growth and during different seasons, the relative growth rate may conveniently be used. Relative growth rate has no direct physiological significance but it can be resolved into two components, the unit leaf rate and the leaf area ratio (Briggs, Kidd and West, 1920; Ballard and Petrie, 1936). In the present paper these conceptions will be used in analysing the effect of the virus on growth.

EXPERIMENTAL METHODS.

Inoculation Technique.

Inoculations were made using freshly expressed virus juice from recently diseased plants. To the juice was added an equal volume of 0·2 p.c. sodium sulphite and fine carborundum was mixed with the inoculum, which was then applied using the spatula method (Samuel, Best and Bald, 1935).

Growth Experiments.

Seeds of the tomato, variety Marglobe, were sown in seed boxes and the seedlings were transferred to 4 inch pots when the first two foliage leaves had appeared. The soil used was a sandy loam to which had been added a little superphosphate. Care was observed in maintaining an adequate water supply to the plants. When the plants had reached the 2–3 leaf stage, they were carefully paired, taking into consideration factors such as height, leaf area and vigour. One group was inoculated on two leaves with T.S.W. virus and the parallel series was treated with distilled water. Both sets of experimental plants were grown under the same conditions in an insect free glasshouse. Immediately after inoculation, ten paired plants were taken at random from the sets and heights, leaf areas and fresh and dry weights determined. Heights were measured from the cotyledon node; comparative values only for leaf area were obtained by Porter's method (1937). Methods somewhat modified from Bolas and Melville (1933) were followed for the determination of fresh and dry weights. Plants were dried at 100° C. for 24 hours. Water content is expressed as a percentage of the fresh weight. There is a range of incubation period

[1] Papers I, II and III of this series appeared in Proceedings of the Royal Society of Victoria.

in tomato plants and therefore a second sample was taken when systemic T.S.W. symptoms were visible in a considerable number of plants. Third and fourth samples were taken during succeeding weeks as the disease ran its course. The average temperature of the glasshouse in which the experiments were conducted was 75° F. in summer and 59° F. in winter (glasshouse heated). For one experiment made in late winter and early spring the glasshouse was unheated and the average temperature was 58° F.

Experiments have also been carried out in an attempt to discover the mechanism of the effect of the virus on growth, particularly in connection with growth-substances. The following methods were used:

(1) The growth-substance content of comparable leaves of paired healthy and inoculated plants was tested on the day of inoculation and after severe bronzing symptoms had commenced to show. Extraction and testing methods used were modified from Overbeek (1938) and Went and Thimann (1937). The *Avena* tests were carried out in a dark room housing a controlled temperature and humidity compartment (Avery *et al.*, 1939).

(2) Control and inoculated members of five to ten paired plants (random sample) were treated immediately after inoculation with a solution of synthetic growth-substance. A further random sample was treated when bronzing symptoms were showing in inoculated plants. β-indole-acetic acid was used, 0·1 c.c. of a 1 in 3,000 or a 1 in 10,000 dilution being allowed to drain into the stem bundles from a small tube drawn out to a capillary at one end. In other experiments the β-indole-acetic acid was applied in lanoline to decapitated plants. Plants were placed under glass cases and observations made on adventitious root formation over a period of 14 to 20 days.

(3) Stem sections (0·5 cm. long), cut from apical internodes of healthy and infected plants, were tested for their effect on β-indole-acetic acid. The polar transport method was used. After the cut ends of the stem parts had been in contact with moist filter paper for 10–15 minutes, they were placed for two hours between agar blocks (12 × standard size). In the upper agar block, β-indole-acetic acid was present in a dilution of 1 in 3 million. The relative amounts of auxin transported through the respective stem segments into the plain agar blocks below was determined by *Avena* test.

The significance of the mean difference between the "paired plants" in the various experiments was estimated according to Fisher's modification of Student's method.

EXPERIMENTAL RESULTS.

The Effect of the Virus on Growth. Résumé of Symptoms.

Before describing the experiments showing the quantitative relations between the growth of diseased versus healthy plants it appears desirable to outline briefly the symptoms of T.S.W. on tomato as observed under glasshouse conditions. Symptoms may be grouped as primary local lesions and as systemic symptoms. In many cases primary local lesions show on leaves which are inoculated. The lesions range from few to many in number and are circular or angular in appearance. They represent the points of entry and of primary multiplication of the virus. Systemic invasion has frequently been found to occur, however, without local lesions becoming apparent on the inoculated leaf. The first systemic symptom is a characteristic reflexing and twisting of leaflets which is shortly followed by the bronzing effect. This bronzing is associated with the development of minute circular to irregular shaped lesions which become visible on the leaves and show rapid increase in size. At this stage they appear watersoaked in appearance, particularly when viewed from the under surface of the leaf, but later they appear brown to black. Further rapid increase of size of these occurs giving angular lesions between the main and lateral veins. Severely bronzed and lesioned leaflets next dry out so that the plant presents a scorched appearance. The disease may cause necrosis of the stem and of all the leaves, but very commonly the apical leaf shows less severe symptoms and frequently only mottling (mosiac effect) is present here. With the onset of bronzing and lesion formation, growth in height is greatly reduced or ceases altogether and if the plant is not killed this check to growth may last for 2 to 3 weeks. After this time, growth in height with the formation of new leaves may be gradually resumed. The newly formed leaves and new growth of those in which lesioning is present show a very definite mottling of the mosaic type. The mottling may take a variety of forms. One of the most characteristic is due to clearing of the young veins and the mosaic effect is more brilliant toward the apex of the leaf. This mottling may also be the only expression of systemic symptoms on inoculation. That mixed viruses are not in question is shown by the fact that in these plants active and sudden development of the typical bronzing and lesioning may occur under changed environmental conditions.

The Dry Weights.

The results of five experiments made over a period of twelve months are summarized in Table 1. The relevant data from this Table are discussed in the following paragraphs.

Fig. 1 shows growth curves for typical experiments at different times of the year, mean dry weights being plotted against time in days after inoculation. The logarithms of the mean dry

weights are also plotted for two experiments. It is to be seen that, after the appearance of symptoms, infected plants either stop growing or grow at a much lower rate as compared with the controls. The season of the year is seen to affect the growth rate and the relative differences which develop between the dry weights of healthy and diseased plants. In the following paragraphs are presented some further observations in relation to the dry weight data of Experiments 1 to 5.

In Experiment 4 (mid-summer, January–February) symptoms were mainly of the bronzing type and a highly significant reduction in dry weight of inoculated plants relative to the controls had occurred by the time the second sample was taken, i.e. fifteen days after inoculation. The majority of the inoculated plants were severely bronzed by the fifteenth day, some more severely than others as 13 out of the 40 test plants showed a minimum incubation period of 8 days.

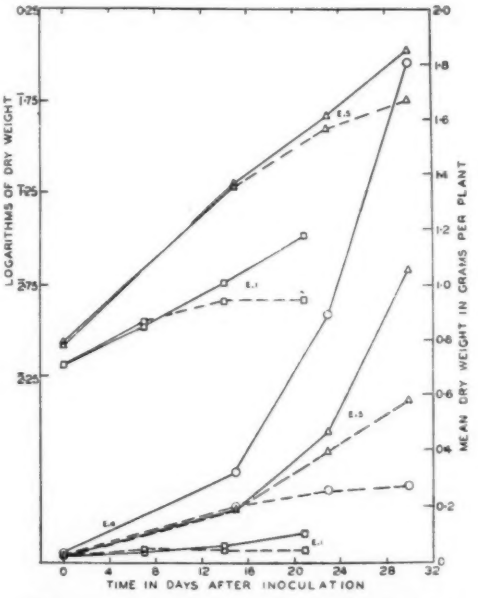

Fig. 1. Typical growth curves for healthy and T.S.W. infected tomato plants. Continuous line healthy plants. Dotted line infected plants. Mean dry weight and logarithms of mean dry weight plotted against time in days after inoculation.

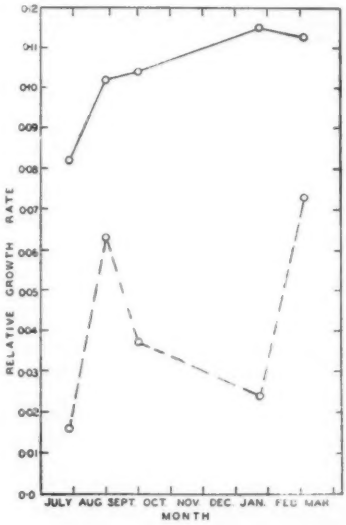

Fig. 2. Curves of relative growth rate and time of year. Continuous line healthy plants, dotted line T.S.W. infected plants. Experiments 1 to 5.

In Experiment 5 (late summer, February–March) the mean incubation period was 14 to 15 days and symptoms were mostly of the light lesion and mottling mosaic type. A highly significant depression in the growth of the diseased plants as compared with the controls, had occurred by the 23rd day of the experiment, or the 7th after the appearance of symptoms in a representative number of plants. In the winter experiments significant differences did not develop until 14 days after the appearance of symptoms. Experiment 1, which was carried out in a heated glasshouse in mid-winter (mean temperature 59° F.), was of unusual interest in that bronzing and severe lesion formation occurred in a high proportion of test plants, after an incubation period as short as that of the summer Experiment 4. The lethal effect of the virus was not as marked as in Experiment 4 however, and within a fortnight new leaf growth showing mosaic symptoms had commenced. In Experiment 2 which was carried out during late winter and early spring when the glasshouse was unheated (mean temperature 58° F.) primary local lesions took almost twice as long to appear as in Experiment 1 and were relatively few in number. Systemic invasion indicated by mottling was mild in character. The incubation period in Experiment 3 (spring) was the longest recorded in these growth experiments, i.e. 22 days. While the symptoms were predominantly of the mottling type, some of the plants showed bronzing.

TABLE 1.

Principal experimental data showing the effect of T.S.W. virus on growth of seedling tomato plants.

Experiment.	Date	Sample 1. T.	L.		Dry weight.	Height in cm.	Leaf area in sq. cm.	Water content.	Sample 2. T.	L.	No. infected.	Dry weight.	Height in cm.	Leaf area.	Water content.
	1941.														
1	July–	0	2·5	I.	0·020	0·83	13·5	92·60	7	3	10/10	0·036	1·2	23·3	*91·42*
	Aug.			C.	0·020	0·82	14·1	92·33		3		0·033	1·2	22·1	92·80
2	Aug.–	0	4	I.	0·042	2·43	33·6	93·53	13	5	7/10	0·109	3·57	50·6	91·83
	Sept.			C.	0·044	2·47	33·2	93·20		5		0·094	3·32	45·9	91·61
3	Sept.–	0	3	I.	0·052	2·9	40·3	92·64	22	(5)	9/10	*0·150*	*5·6*	*80·5*	*92·50*
	Oct.			C.	0·052	3·0	41·1	92·58		6		0·208	7·0	106·0	92·88
	1942.														
4	Jan.–	0	3	I.	0·028	3·3	22·4	95·14	15	(5)	8/10	*0·200*	*10·6*	*86·9*	*93·14*
	Feb.			C.	0·030	3·2	23·5	94·81		5·5		0·322	14·8	133·5	93·36
5	Feb.–	0	2·5	I.	0·027	1·6	18·5	93·23	15	5	5/10	0·193	7·3	83·6	*93·75*
	Mar.			C.	0·026	1·4	18·4	93·50		5		0·194	7·5	95·0	94·35

Experiment.	Date	Sample 3. T.	L.	No. infected.	Dry weight.	Height in cm.	Leaf area.	Water content.	Sample 4. T.	L.	No. infected.	Dry weight.	Height in cm.	Leaf area.	Water content.
	1941.														
1	July–	14	3·5	10/10	0·044	1·7	—	*88·93*	21	(3·5)	10/10	*0·045*	*1·8*	—	*87·42*
	Aug.		3·5		0·057	2·1	31·7	91·84		4·5		0·104	3·2	—	92·09
2	Aug.–	20	6	8/10	*0·155*	5·07	*63·0*	*91·44*	27	(6·5)	8/10	*0·265*	6·81	73·5	90·88
	Sept.		6		0·195	5·72	87·1	91·87		7		0·389	6·96	89·9	91·00
3	Sept.–	28	(6)	9/10	*0·182*	*5·8*	*75·2*	*88·91*	—	—	—	—	—	—	—
	Oct.		7		0·387	7·9	131·3	91·60							
	1942.														
4	Jan.–	23	(5·5)	9/10	*0·254*	*10·5*	*95·5*	*92·03*	30	(5·5)	5/5	*0·274*	*10·8*	—	*90·63*
	Feb.		8		0·883	24·2	301·6	92·64		9·5		1·803	30·5	—	92·47
5	Feb.–	23	7	10/10	*0·389*	*11·0*	*141·7*	93·13	30	(7·5)	5/5	*0·578*	*14·4*	—	92·07
	Mar.		7		0·467	13·0	182·3	93·29		8·5		1·052	19·0	—	92·41

I = inoculated plants, C = controls, T = time in days after inoculation and L = leaf number. Where the value of any attribute of the inoculated series is significantly less than that of the control, that value is placed in italics.

The Leaf Areas.

Leaf area data are presented in Table 1. Owing to the difficulty of estimating leaf area in infected plants when symptoms become severe, comparative data are given only to sample 3 in four of the experiments. Bronzed and heavily lesioned leaves fail to grow in size, while frequently shrinkage occurs. At sample 3, significant differences were present between the leaf areas of control and infected plants in all experiments. This disparity in leaf areas was very obvious on inspection at sample 4, except in the case of Experiment 2 where the depression in leaf area had passed off.

The regions of development of lesions on leaves of systemically invaded plants are of interest in relation to leaf area data. Lesions commonly occurred in "blocks" either at the apex, the base or across the centre of leaflets. In young apical leaves the "block" of lesions was generally toward the apex. Leaf area development was hindered most when lesions were concentrated toward the basal portions of the leaflets. Increase of leaf area recommenced when the lesioning phase was succeeded by the mottling phase of the disease.

The Relative Growth Rate (R).

To compare the effect of the virus on the growth of the plant from the time of inoculation to the conclusion of the experiment, the relative growth rates were calculated (see Ballard and Petrie, 1936) being given in Table 2. The relative growth rates will be considered over the incubation period and over the period during which symptoms were present.

The relative growth rate during incubation period. The incubation period may be defined as that time which elapses between inoculation and the appearance of symptoms. In T.S.W. in tomato the virus multiplies at the point of inoculation often producing local lesions there. It then spreads through the plant generally producing bronzing and sometimes mottling symptoms.

TABLE 2.

Relative growth rate, unit leaf rate and leaf area ratio.

I = inoculated plant series, C = control series. Significantly different values are in italics.

Experiment.	Sample.	1–2	2–3	3–4
Relative growth rate (R)				
1	I.	0·084	0·029	*0·003*
	C.	0·071	0·078	0·085
2	I.	*0·073*	*0·050*	0·076
	C.	0·058	0·104	0·098
3	I.	0·048	*0·037*	—
	C.	0·062	0·103	—
4	I.	*0·131*	*0·030*	*0·015*
	C.	0·157	0·126	0·106
5	I.	0·130	*0·088*	*0·056*
	C.	0·134	0·110	0·116
Leaf area ratio (F_A)				
2	I.	558	430	325
	C.	573	460	303
4	I.	480	402	—
	C.	446	361	—
5	I.	464	387	—
	C.	516	420	—
Unit leaf rate (100 E_A)				
2	I.	0·0126	0·0116	0·0230
	C.	0·0100	0·0220	0·0315
4	I.	0·0240	*0·0073*	—
	C.	0·0306	0·0340	—
5	I.	0·0251	0·0224	—
	C.	0·0230	0·0251	—

The range for individual incubation periods in a group of 40 or more inoculated plants is often about one week, consequently the taking of the second sample was deferred until a representative number of plants were showing symptoms. Minimum incubation periods for individual plants in each experiment were also recorded. Study of these, together with the mean incubation periods in relation to growth rate and time of year, showed no relation between incubation period and the rate of growth of the plants at different seasons. Thus in Experiment 1 (winter) the mean incubation period was short (mean 7 days, minimum 6 days), symptoms were of the severe bronzing type and the value of R (Table 2) was 0·084. In Experiment 4 (summer) the mean incubation period was considerably longer (mean 15 days, minimum 8 days) and the value of R was 0·131, i.e. 1·5 times as great as in Experiment 1. The fact that minimum incubation periods are much the same in Experiments 1 and 4 indicates that the time taken for the virus to travel from the point of inoculation throughout the plant may be the same in summer and in winter, i.e. it is independent of the growth rate. The variation in the incubation period in individual plants both in the summer and in the winter also indicates that some factors other than growth rate are concerned with rate of spread of the virus.

In Experiments 1 and 2 (winter) the R values of inoculated plants during the incubation period are slightly higher than those of the controls. The differences are significant only in the case of Experiment 2, but both experiments suggest that the virus has stimulated growth during the incubation period. The effect does not show in Experiments 3, 4 and 5. For further studies on this point it appears desirable to use minimum incubation periods in sampling.

The relative growth rate after the appearance of symptoms. To compare the relative growth rates of healthy and of infected plant series at different times of the year it is desirable that these rates be calculated over comparable stages of morphological development. This however, was not possible in all cases and the R values are compared over the periods corresponding to the second and fourth sampling periods (see Fig. 2). R values for infected plants over this period are invariably lower than for controls and it is also apparent that the season has a very definite effect on growth.

The relative differences between the two sets of growth rates, however, do not appear to be related to the season. This is also shown when the growth rates of virused plants are calculated as a percentage of the controls.

Leaf Area Ratio (F_A).

The leaf area ratio data are presented in Table 2. Statistical analysis failed to show any significant difference between the paired values for control and infected plants.

Unit Leaf Rate (E_A).

Unit leaf rates are given in Table 2. A significant reduction of E_A was found in Experiment 4. In Experiments 2 and 5 the value for E_A in infected plants was less than that of the controls, but the difference could not be shown to be significant.

F_A and E_A as Determinants of R.

Since R is the product of F_A and E_A the data in Table 2 may be examined to ascertain the relation of these components to the depression of R in infected plants. In Experiment 4 the depression of R is attributable solely to the depression in E_A. In Experiments 2 and 5 although statistical analysis showed no significant depression of E_A for diseased plants, it may be noted that in both cases the value of E_A for such plants had fallen below that of the controls.

The difficulties of estimating leaf area in infected plants as symptoms develop, precluded analysis of F_A and E_A beyond sample 3. The use of the analagous quantities F_W and E_W (Ballard and Petrie, 1936) for future work is indicated. Lack of the necessary additional facilities and labour prevented the separate determinations of leaf weights in the present investigation.

The data given in Table 2 are sufficient to indicate, however, that the virus probably exerts its main effect on R through the depression of the net assimilation rate.

Starch Metabolism.

Since in typically bronzed plants there was a reduction in relative growth rate and since the evidence suggested that this was due to a fall in the net assimilation rate (E_A), it appeared that an examination of the starch relations of healthy and infected leaves would be of value in the further elucidation of the effect of the virus on growth. Accordingly leaves showing initial bronzing, lesions and mottling mosaic were collected at the end of a sunny day, decolorized, and stained in a solution of iodine in potassium iodide. Typical results are illustrated in Fig. 5. In young plants tested when bronzing symptoms were just commencing, starch formation was not noticeably depressed as compared with the controls. At the acute bronzing and lesioning phase, some 2 to 3 days after the first appearance of symptoms, starch was absent from the lesioned areas and the density of staining in other parts of the infected leaf was much less than in a control leaf. When the active bronzing phase was succeeded by the mottling phase, the iodine test showed less starch in the mottled leaf than in the control. Fig. 5 B, shows a leaf in which both lesions and mottling are present. Starch is absent from the lesioned areas and shows an irregular pattern with some concentration along the veins in the mottled part. Evidence of overnight delay in hydrolysis of starch was also obtained.

It is clear from these experiments that T.S.W. virus depresses starch formation and delays its hydrolysis. It is very likely that these effects on starch metabolism contribute to the depression of growth. For further understanding of the effect of the virus on growth an investigation of carbohydrate metabolism and of respiration is, however, required.

Water Content.

In all five experiments, after the appearance of symptoms, whether of the lesion or mottling type, the percentage water content of infected plants fell significantly below that of the controls (Table 1). In Experiment 5 (symptoms mainly of the mottling type) the water content rose again 7 to 8 days after the appearance of symptoms, samples 3 and 4 showing no significant differences between the pairs. The fall in percentage water content in infected plants developing severe lesions was to be expected. The mean differences are most striking in Experiment 1 (winter)

although symptoms were rather less severe here than in Experiment 4 (summer). In Experiments 2 and 5 the fall in water content consequent on the appearance of the mosaic type of symptoms is similar to that found by Ainsworth and Selman (1936) in the case of tobacco mosaic virus infection on tomato. They also found in such plants that at a later stage in some experiments the percentage water content rose above that of the controls. While there was evidence of a rise in water content relative to the controls in three of the T.S.W. experiments reported, in no case did the water content of infected plants become higher than that of the controls. The importance of water content in relation to assimilation in tomato plants has been stressed recently by Melville (1937) and the fall in water content of both lesioned and mottled plants initiated by the virus could contribute to the depression of assimilation.

The Relation between Growth Rate and Symptom Expression.

From the data available, it is now possible to examine on a quantitative basis, the general observation that there is a relation between symptom expression and vigour of growth in tomato (Bonnemaison, 1939). The author's experiments and those of other investigators (Ainsworth and Selman, 1936; Ashby, 1937 and Luckwell, 1937) show that the relative growth rate is highest between the period of development of the 3rd and 6th leaves. The decline in growth rate which sets in by the time the 6th leaf has developed continues up to the development of the first flower truss (Ashby, 1937) and on through the flowering period (Luckwill, 1937). Tomato plants are very susceptible to T.S.W. when inoculated at the 2–3 leaf stage. Severe bronzing symptoms generally appear by the time they are approaching the 4–5 leaf stage. The occurrence of this severe bronzing appears therefore to be associated with high growth rate in young plants at the 2–5 leaf stage. When the relative growth rates of leaves only, at the different stages of morphological development of the plant, are calculated (data of Ashby, 1937, and of Luckwill, 1937) the same relation is found to hold. This is perhaps more important than the agreement for total dry weight growth, since the leaf is the unit mainly concerned in virus multiplication and the part in which symptom expression mainly occurs.

In pot-bound plants or in those growing under unfavourable conditions, it is likely that the relative difficulty of infection and the mildness of symptoms (mottling type) is related to a lower relative growth rate in such plants. The change from bronzing to mottling symptoms in new growth of plants also seems most reasonably explained by reference to growth rate.

Certain facts accumulated in the course of this investigation indicate, however, that factors other than growth rate are also concerned with symptom expression. When the logarithms of the dry weights of healthy tomato plants are plotted against time (Fig. 3) the curves approximate to straight lines and the slopes may be taken as measures of the rate of growth. It is apparent that the greatest growth rate occurs in the summer and the lowest in the winter months. If the relation between growth rate and severity of symptoms held, one would expect bronzing in the summer and mosaic mottling in the winter. This was not found to be the case in certain of the author's experiments (cf. Experiments 1 and 4, Table 2). Similarly the variation in symptoms experienced in experiments using batches of plants of comparable size and vigour at the 3–5 leaf stage cannot be ascribed to differential growth rates. Again the rapidity with which local lesions may develop on leaves previously showing mottling, when a cool change follows a period of warm weather, suggests the operation of a more specific factor than growth rate.

In the interpretation of these and other exceptions, the operation of the following two factors may be considered: (a) change in virulence of the parasite, and (b) change in metabolism or physiological condition in the plant which either facilitates or depresses the multiplication of the virus. Evidence for temporary attenuation of the virus is given by experiments in which juice from long-infected plants is inoculated into susceptible rapidly growing plants. Mild symptoms only occur (see also Bonnemaison, 1939). Again when juice from young recently diseased plants showing only mottling symptoms is inoculated to fresh young plants, mottling symptoms only result. On the other hand the severe bronzing of infected plants in Experiment 1 (winter) is believed to be due to increased virulence arising out of constant transfer of the virus in young tomato plants. The state of the virus may also be influenced by environmental conditions. After a change from warm to cool, dull weather, mottling may rapidly be replaced by lesion formation. This suggests that the change in environment affects some phase of the host metabolism which renders the virus active.

Best (1936) has made an interesting suggestion regarding the effect of light on metabolism in relation to local lesion formation by T.S.W. in tobacco. He considers it likely that, under the more intense reducing conditions prevailing in the cells at low light intensities (due to lowered oxygen tension in cells when respiration exceeds photosynthesis), the multiplication of the virus is facilitated. This explanation cannot apply in the case of systemic infection by T.S.W. in tomato, since experiments in which the light intensity was reduced by shading and capping of infected plants failed to bring about the change from mottling to lesion symptoms. The fall in temperature or the interaction of lowered light and temperature would appear to be more important. It therefore appears that knowledge of the causes of the very variable incubation periods in any experiment, and of the variable symptom expression, would be advanced by studies on the metabolism of the tomato plant under various controlled combinations and variations of temperature and light.

The Height Data.

Data on height for the five experiments are given in Table 1. Growth curves for height in typical series are presented in Fig. 4. The effect of the seasons of the year is reflected again in the curves. Diseased plants show significant stunting in height as compared with the controls in all but one experiment. The effect of the virus in Experiment 4 (midsummer) was to cause almost complete cessation of growth in height for 15 days after the appearance of systemic bronzing. This experiment was concluded with only a suggestion of recommencement of growth in height in the affected plants. Longer continued observations on height of virused plants in a parallel subsidiary experiment showed that the inhibition of growth in height persisted for some two to three weeks after bronzing. Slow growth then commenced in those plants in which the

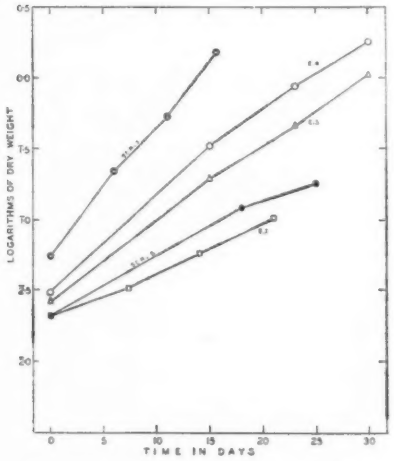

Fig. 3. Logarithms of total dry weight of healthy seedling tomato plants plotted against time in days. Data from Table 1. Circles, Experiment 4 (summer); triangles, Experiment 5 (summer); squares, Experiment 1 (winter). Data from Ainsworth and Selman (1936). Double circles, Series 1 (summer); black circles, Series 5 (winter).

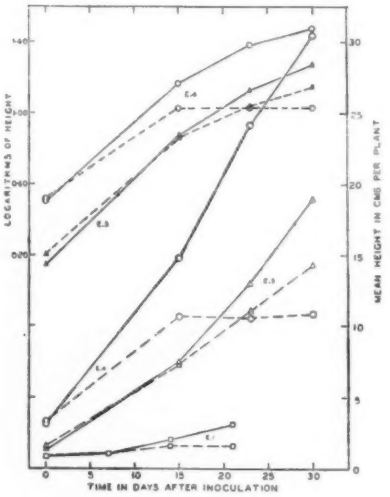

Fig. 4. Typical growth curves as height for healthy (continuous line) and T.S.W. infected (dotted line) seedling tomato plants. Height and logarithms of height plotted against time in days after inoculation.

apical growing point had survived. In Experiments 1 and 3 (July–August and September–October respectively) the stunting effect occurred as in summer but was less severe. The new growth, even in leaves partially covered with lesions during the systemic expression of the disease, was of the mottling type. In Experiments 2 and 5 where lesion formation was transient, mottling being the main symptom, the retarding effect was less in evidence.

In Experiment 2 no significant depression in the rate of growth in height occurred although some retardation relative to the controls was apparent in sample 3. This effect, however, appeared to have passed off by the time sample 4 was taken. The result for this experiment suggested testing the effect of T.S.W. virus attenuated by age, on growth in height. Accordingly one set of paired plants at the two-leaf stage and another at the four-leaf stage at the time of inoculation were selected. The resulting symptoms were completely and continuously of the mottling type. Statistical examination of the data (Table 3) showed no significant difference between the heights of the inoculated and control plants during the experiment.

The existence of a distinct trend towards greater height in the inoculated plants in the post-incubation period may, however, be noted.

In contrast therefore to the "active" virus which temporarily inhibits growth in height, the attentuated or "latent" form, is not associated with any depressant effect.

The question as to how the "active" virus causes the sudden cessation or marked reduction of growth in height is a matter of considerable interest. The operation of the factors depressing growth as dry weight which were considered earlier in this paper, do not appear to be sufficient to explain it. The possibility of interference by the virus with the growth hormone mechanism was therefore examined.

TABLE 3.

Showing the effect of T.S.W. virus (latent form) on growth as height in seedling tomato plants.

T = time in days after inoculation, Ht = height in cm., n.s. = no significant difference, + = difference in favour of inoculated plants.

		T.	Ht.	T.	Ht.	T.	Ht.	T.	Ht.	T.	Ht.	T.	Ht.	T.	Ht.	T.	Ht.
Expt. 1	Inoc.		1·4		2·3		3·6		5·6		8·3		10·0		12·1		15·8
		0		3		6		9		12		14		18		23	
	Control		1·5		2·4		3·8		5·9		8·7		9·7		11·7		14·6
								(End of incubation period)									+
	Mean Diff.																1·2 ±0·9 n.s.
Expt. 2	Inoc.		3·7		5·0		7·8		11·7		14·9		16·3		18·7		
		0		3		6		9		12		14		18			
	Control		3·7		5·4		8·1		11·2		14·0		15·4		17·6		
															+		
	Mean Diff.														1·1 ±0·9 n.s.		

The Effect of the Virus on Plant Growth Hormone.

Auxin assay. Non-diffusible, ether extractible auxin is regarded as being essential in addition to food materials and water for the enlargement of newly formed cells (Went, 1942). It is produced in leaves and transported to regions of development where it promotes normal growth. Results of assays of hormone in comparable leaves from paired inoculated and control plants at the 3–5 leaf stage are given in Table 4.

TABLE 4.

Auxin assay of comparable leaves of healthy and infected seedling tomato plants. Concentration in degrees curvature. Each figure is a mean of 6 to 10 Avena *curvatures. Significantly different values are in italics.*

Experiment No.		1	2	3	4	5	6	7	8	9
Time in days after inoculation		0	0	0	0	7	9	8	9	11
						Inoculated plants showing severe bronzing.				
Degrees of Curvature	Inoc.	6·8	8·5	9·5	5·8	*3·4*	*2·8*	*0·0*	*2·4*	*3·0*
	Control	5·9	9·1	9·9	5·7	9·1	11·2	7·7	6·7	22·0

These assays were made either immediately on inoculation or at the time when the leaves were showing bronzing and lesion formation.

The experiments using leaves from paired healthy tomato seedlings showed approximately the same amounts of auxin present, no significant difference being found. In the experiments performed after the appearance of bronzing and lesion formation a significantly smaller amount of auxin was extracted from infected leaves as compared with the controls (see Fig. 7). As the plants were originally paired for size and vigour, and as leaves at the same stage of morphological development were used for extraction, the results give a clear indication that auxin is destroyed or its production depressed in bronzed leaves. It was found on testing apparently unaffected leaves (i.e. those below the point of inoculation) that auxin was present in normal amounts. Methods were therefore developed to test the likelihood of the breakdown of this auxin before it reached regions of development.

Reaction to synthetic growth-substance. Paired plants were treated with β-indole-acetic acid and their reaction, as judged by adventitious root formation, recorded. Results, representing an extension of those reported earlier (Grieve, 1936), are presented in Table 5. Significantly fewer adventitious roots were found to develop on the diseased plants than on healthy controls.

TABLE 5.

Showing the reaction (by adventitious root formation) of paired control and T.S.W. inoculated tomato plants to β-indole-acetic acid. Concentration 1 in 3,000 for Experiments 1 and 6 and 1 in 10,000 for Experiments 2 to 5. Significantly different values are in italics.

Experiment No.		1	2	3	4	5	6
No. plant pairs		5	5	9	10	5	5
Time in days after inoculation		0	0	10	14	8	8
				(light bronzing)		(severe bronzing)	
No. of adventitious roots	Inoc.	57·2	14·5	10·0	*7·7*	*5·6*	*37·0*
	Control	55·4	15·1	13·0	15·7	16·0	92·4

Qualitative differences in degree of outgrowth of adventitious roots and in cambial activity (as shown by stem swelling), which were also present between healthy and infected plants used in these experiments, are illustrated in Fig. 6. The healthy plants may be seen to have more strongly developed adventitious roots and evidence of their greater cambial activity may be seen clearly in Fig. 6c.

Polar transport of synthetic growth-substance. In basipetal transport experiments using β-indole-acetic acid, the *Avena* tests showed that significantly less growth-substance (approximately half as compared with the controls) had passed through the infected stem parts into the agar blocks below (see Table 6).

TABLE 6.

Basipetal transport of β-indole-acetic acid (concentration 1 in 3 million) through infected and healthy stem segments (0·5 cm. long). Each figure is a mean of 6 Avena *curvatures.*

Experiment No.	1	2	3	4	5	Mean.
Inoc.	2·8	3·0	8·0	6·0	4·5	4·9 ± 0·9
Control	11·2	13·4	12·5	8·0	11·5	11·3 ± 0·9

It is concluded from the above experiments that in T.S.W. infected plants during the bronzing phase, added growth-substance is partially destroyed. These results support and extend those found in the assays of plant auxin in healthy and infected plants.

The experiments make it possible to conclude that the virus acts in two ways—firstly by reducing the amount of auxin in bronzed leaves and secondly by inactivating auxin during translocation to the growing point. Since auxin is necessary for cell elongation, its inactivation in greater or less degree in infected plants must be considered an important factor in relation to inhibition of growth as height.

DISCUSSION.

The results given in this paper give quantitative expression to observations by earlier workers (Samuel *et al.*, 1930; Bald and Samuel, 1931) on the growth-depressing effect of T.S.W. virus in tomato. Significant reduction in the growth in dry weight, height, and leaf area of infected plants rapidly occurs when the symptoms are of the usual bronzing type. Mottling symptoms, however, are accompanied by a less serious depressant effect of much the same order as that reported by Ainsworth and Selman (1936) for tobacco mosaic virus in tomato. The growth rate of T.S.W. inoculated plants relative to the controls is maintained during the incubation period. Under the conditions of these experiments there is no relation between the duration of incubation of the virus and the growth rate of the plants. The possibility is not excluded, however, that if experiments were carried out in mid-winter in an unheated glasshouse at Melbourne, a longer incubation period would be associated with the very slow growth. Some relation might also be expected between falling growth rate in older plants, incubation period and symptom expression. A relation between age and incubation period has been recorded by Bald (1937). He showed that during the early growth in tomato there is a well defined 12-day incubation period for T.S.W. after vector infection. With the change from youth to comparative maturity, the incubation period was extended by variable amounts. The symptoms at this time were also milder.

The author's experiments in which older glasshouse grown plants were inoculated at the flowering to fruit setting stage also indicated this relation.

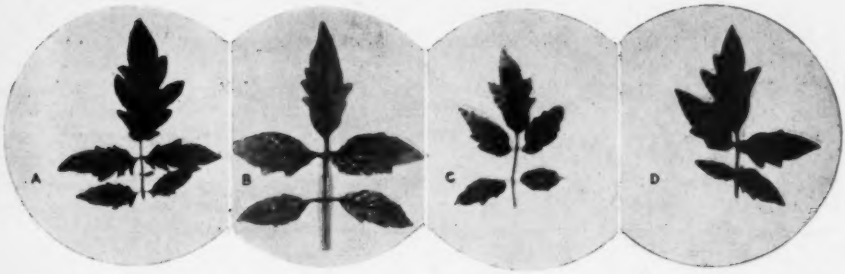

Fig. 5. Starch prints of leaves of tomato plants infected with T.S.W. virus (A = mottled slightly lesioned leaf; B = leaf with mottling symptoms, lesioning towards apex; C = leaf with bronzing and lesioning (black spots represent necrotic areas); D = healthy leaf. Note contrast in starch formation between control and infected plants.

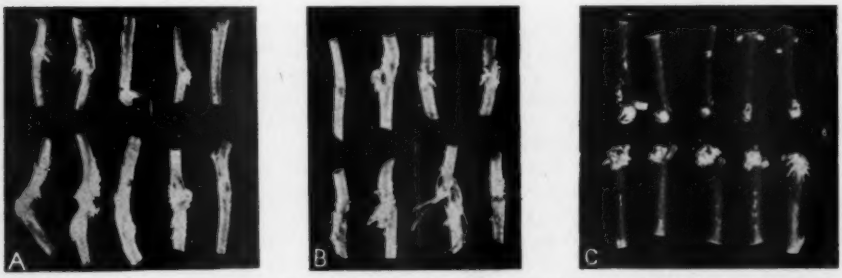

Fig. 6. Showing the reaction of T.S.W. infected (upper) and control plants (lower) to β-indole-acetic acid (1/10,000 concentration). A, B = stem uptake method, C = lanoline smearing method. Note greater numbers of adventitious roots and greater stem swelling on controls.

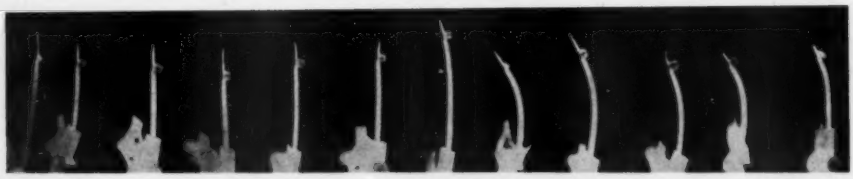

Fig. 7. Showing the result of an auxin assay on infected leaves (6 coleoptiles on left) and healthy leaves (6 coleoptiles on right) of seedling tomato plants. The smaller amount of auxin present in lesioned leaves as compared with the controls is reflected in the difference in curvature of the coleoptiles.

Ainsworth and Selman (1936) reported in the case of tobacco mosaic virus in tomato that there was a high negative correlation between the growth rate of the seedling plants and the incubation period. This result was (as they point out) only to be expected, because only those leaves developed after infection show symptoms. In T.S.W. in tomato on the other hand, bronzing or mottling symptoms do not necessarily appear as a growth response, since they develop on most leaves present at the time of inoculation as well as on those formed later. The variability of the incubation period for symptoms in any one batch of experimental plants, all inoculated at the same time with the same inoculum, is believed to be brought about by slight differences in metabolism between individual plants. Differences in virulence between inocula used are responsible for consistent differences in symptom type when comparable batches of plants are infected.

The cause of bronzing as against mottling appears to be related to growth rate, host metabolism under changing environmental conditions and to altered virus activity. Hildebrand and Curtis (1942) have recently described the use of a darkening technique for inducing Yellow-Red disease of peach, in mature as well as in growing leaves. They are of the opinion that the darkening of the leaves favours the transport of carbohydrates into the shaded part and that the virus is carried along with the entering food. This explanation of symptom development does not apply in the case of T.S.W. When symptoms of T.S.W. in tomato are of the mottling type there is no question of movement of virus into a given leaf; it is already present, but with the change of environment the type of expression changes from mottling to lesion formation. The work of Hildebrand and Curtis does, however, indicate the importance of metabolism in relation to symptoms. One may make the suggestion in the case of T.S.W. that perhaps some change in the rate of movement of carbohydrate or its availability is concerned.

The reduction in growth as dry weight and the inhibition of growth in height and of morphological development in plants showing bronzing symptoms, appear to be due to the partial destruction and reduction in efficiency of assimilating tissue and to destruction of growth hormone. Tissue breakdown in bronzed leaves involves the destruction of plant hormone. Experiments on the reaction of infected and healthy plants to β-indole-acetic acid and the diffusion experiments indicate that the virus may exercise a destructive effect on hormone in parts of the plant other than bronzed leaves. The question as to whether the virus itself, or some substance present in infected plants arising out of virus infection, is responsible for this inactivation of hormone is an interesting one. Best (1937) reported the presence of an "oxidase" enzyme in the juice expressed from T.S.W. infected tomato plants. As oxidases are known to destroy auxins, it appears probable that the virus indirectly acts on plant growth hormone through this oxidase enzyme.

Practical Suggestions.

It has been pointed out that milder symptoms are associated with increasing maturity and falling growth rate in tomato plants (var. Marglobe) grown in a glasshouse. The author's experience at Melbourne is, that under glasshouse conditions, yield is not obviously affected by T.S.W. infection once the fruit has set. From this it is suggested that in glass houses, spraying with tartar emetic for example (see Magee *et al.*, 1942) need only be carried out from seed box to setting of fruit and not to fruit ripening. One or two additional sprayings could be given when vegetative growth is resumed after picking of the first crop.

Whether a shortened spray schedule in the field would be justified is a matter for experiment as it is manifestly unsafe to make predictions from glasshouse results. The desirability of testing the suggestion however, is indicated by some data of Bald (1937). He noted that after 10 to 12 weeks or alternatively at the

time of fruit setting, there was in the field a reduction in growth which extended until the time of ripening of the first crop and that a longer incubation period and milder symptoms were found over this period.

SUMMARY.

The effect of tomato spotted wilt virus on the growth of seedling tomato plants has been examined. Bronzing symptoms have been shown to be associated with a significant depression in dry weight, height, leaf area, leaf development and in water content of infected plants relative to the controls. Mottling symptoms are accompanied by a less serious depressant effect on dry weight and growth as height was unaffected.

Evidence is presented to show that the virus exercises its effects on growth in dry weight, at least in part, by partial destruction and reduction in efficiency of assimilating tissue and on height by destruction of growth hormone.

Under the conditions of the author's experiments no relation was found between the rate of growth of the seedling plants and the incubation period for the virus. Analysis of data from growth and infection experiments indicated that factors such as growth rate, host metabolism and relative virus activity conditioned symptom expression.

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HAEM-AGGLUTINATION BY VIRUSES

THE RANGE OF SUSCEPTIBLE CELLS WITH SPECIAL REFERENCE TO AGGLUTINATION BY VACCINIA VIRUS.

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The specific agglutination of chicken erythrocytes by allantoic fluid from chick embryos infected with influenza virus was first described by Hirst (1941) and a little later independently by McLelland and Hare (1941). The reaction has already been widely applied to various aspects of influenza virus research (Hirst, 1942; Burnet, 1942a; Burnet *et al.*, 1942) while in addition to influenza virus it has been shown that Newcastle disease virus (Burnet, 1942b), vaccinia virus (Nagler, 1942) and fowl plague virus (Lush), show similar specific agglutinations of certain types of red cells. The present contribution provides data on the range of animal species whose red cells are susceptible to the various types of virus.

Technique.

Red cells. Blood was collected into tubes containing 4 p.c. sodium citrate solution, the cells spun down and washed twice with saline. The packed cells were then made up in saline to a 2 p.c. suspension.

Agglutination tests were carried out according to the method described by Burnet (1942a). Hirst's standard method (1942) was considered inapplicable in an investigation in which we were interested in minor degrees of agglutination as well as the typical Hirst reaction with influenza virus and chicken cells. In brief the method makes use of a total volume of 0·75 ml. made up of one volume (0·25 ml.) diluted virus, one volume of saline (or serum dilution) and one volume of 2 p.c. red cells. For influenza and Newcastle disease viruses the tubes were kept at refrigerator temperature until the cells in control tubes with saline only had settled to the bottom in a compact lenticular deposit. The reading was made in terms of the pattern of agglutinated cells over the bottom of the tube.

Vaccinia virus tests were made by Nagler's method using a smaller total volume (0·5 ml.) and keeping the tubes at 36° C. for one hour before reading.

Virus strains. The influenza strains were the standard types W.S., "Melbourne", "Swine 15" and "Lee" (B) used in the form of allantoic fluid from infected embryos. The Newcastle disease virus, also in the form of allantoic fluid, is the Australian strain of Albiston and Gorrie (1942). Vaccinia virus was prepared from chorioallantoic membrane lesions as previously described. The strain was the standard vaccine-lymph strain of the Commonwealth Serum Laboratories. We would stress our previous observation that calf lymph either fresh or glycerolated is almost inactive as an agglutinating agent; chorioallantoic material only must be used. Preparations similar to vaccinia were made from fowl pox and herpes (H.F. strain) chorioallantoic lesions. These were tested against most of the red cell species with essentially negative results, only a few equivocal clumps being seen with low virus dilutions. These two viruses are therefore excluded from the Tables.

Susceptibility of Different Types of Red Cells.

The general results are shown in Table 1, giving the readings obtained when a 1: 10 dilution of the virus shown was mixed with one volume of saline and one volume of red cells. In most instances blood from several individuals of each species was used. Where differences in individual samples were observed these are indicated.

It is naturally impossible to indicate in a single Table all the qualitative and quantitative differences in the reactions observed and we would stress the considerable degree of variation observed, particularly in regard to the weaker reactions. The titre and freshness of the virus preparation used and probably some unrecognized factors can make rather large differences in the results when only a single dilution is used and the Table should be taken as providing a broad general picture of the situation and not as being reproducible in every detail.

In regard to the influenza-Newcastle disease group the outstanding features were first the wide reactivity of B (Lee) which with the exception of the bloods from 6 of 13 horses and one of two donkeys agglutinated all the red cells tested. The influenza A strains were all notably weak against most types of mammalian cells with the exception of those of man and guinea-pig. Where positive results are shown with other species the deposit usually took the form of a very finely granular sheet forming a coherent layer over the bottom of the tube and not readily slipping toward the centre like the rapidly agglutinated cell types. Mammalian cells deposit more slowly than those of the other orders and with some species nearly three hours were needed for complete deposition.

TABLE 1.

Red blood cells from	Influenza B. "Lee".	Influenza A. W.S.	Influenza A. "Melbourne".	Influenza A. "Swine".	Newcastle disease.	Vaccinia.
Human	+++	+++	+++	−	++	+
Sheep	+++	−	++	−	+++	±, −
Goat	+++	−	++	−	+++	±, −
Cow	+++	−	−	−	+++	+, −
Pig	+++	−	−	−	−	+
Horse	++, −	−	−	±, −	−	−
Donkey	++, −	−	−	+, −	−	−
Cat	+++	−	−	−	−	+
Ferret	++	++	++	−	−	+
Rabbit	++	++	++	++	+	+, −
Guinea-pig	+++	++	++	−	+++	+, −
Rat	++	++	++	−	+++	+, −
Mouse	++	++	++	−	+++	+
Ringtail opossum	+++	−	−	−	++	−
Marsupial mouse	++	++	++	++	−	−
Hen	+++	+++	+++	+++	+++	+++, −
Duck	++	++	++	++	++	++
Sparrow	+++			+++	−	−
Frog	+++	+++	+++	+++	+++	−
Tiger snake	+++	+++	+++	+++	+++	−
Copperhead snake	+++	+++	+++	+++	+++	+
Bluetongue lizard	+++	+++	+++	+++	+++	−

Notes. All viruses were used at a dilution of 1: 10 (final dilution 1: 30).

Swine influenza virus used was less active than some other samples of this virus: guinea-pig cells are usually agglutinated at this dilution.

+++ complete typical agglutination. ++ complete slow agglutination with uniform layer of agglutinated cells. +, ± partial agglutination.

Birds, reptiles and amphibians all provide cells which are rapidly agglutinated by all the influenza virus strains used in this work.

The survey indicates that for this group of viruses fowl cells are the most generally satisfactory, with guinea-pig and human cells the only mammalian types that can be usefully employed. It will be noticed that the Table gives the impression that there are three groups amongst the five strains used, B, the three A strains, and Newcastle Disease. The "Swine" strain virus used was of rather low titre and more active samples would have given a closer resemblance to the "Melbourne" and W.S. results.

The vaccinia results give a completely different picture. About half the individual fowls tested gave highly susceptible cells, the others gave cells which were inagglutinable or showed mere traces of agglutination at low dilutions. No other species gave cells agglutinable to more than a tenth of the titre obtained with satisfactory fowl cells. It was difficult to be certain whether some of the apparent agglutinations with other types of cell were or were not specific. Most were inhibited by normal as well as by immune sera and with some no obvious absorption of agglutinating agent occurred. It was obvious that of all those tested fowl cells of correct type were the only ones of any practical value.

The Agglutinability of Fowl Cells by Vaccinia Virus.

It has already been reported that only certain fowls provide suitable red cells for the agglutination reaction. Through the co-operation of Mr. J. Warren of Ferntree Gully, we had an opportunity to investigate the distribution of agglutinable cells in a fairly large number of indi-

vidual birds including groups of varying age and sex and of two different breeds, white Leghorns and black Australorps. In Table 2 the results of examining 78 fowls are shown. The cells from each bird were tested with 1: 5 dilution of stock vaccinia virus which gave an end point of 1: 128 with hen cells of known susceptibility. Cells giving a +++ reaction were taken as positive; the others either gave no agglutination or a weak ± reaction and were classed as negative.

TABLE 2.

Distribution of agglutinable and inagglutinable cells in 78 fowls.

Age of birds.	Breed.	Sex.	Number of fowls with Agglutinable cells.	Inagglutinable cells.
6 months	B.A.	M.	3	7
6 „	B.A.	F.	6	3
6 „	W.L.	M.	6	4
6 „	W.L.	F.	7	3
12 „	Crossbred	F.	3	7
18 „	B.A.	F.	8	2
18 „	W.L.	F.	6	4
30 „	W.L.	F.	4	5
			43 (55%)	35 (45%)

From the Table it will be seen that irrespective of age, breed and sex there are approximately 50 p.c. of birds with agglutinable and 50 p.c. with inagglutinable cells. Any further investigation would obviously call for a study of the inheritance of the two qualities but we have had no opportunity to undertake such work.

As is well known, embryonic chicken erythrocytes are fully agglutinable by most influenza virus strains. Cells from 50 of our stock white Leghorn embryos were all inagglutinable by vaccinia virus. A few of these eggs were allowed to hatch and 3 of 9 chicks were found to give agglutinable cells at the age of two weeks. A larger group of one day old cockerels was obtained from a breeder of white Leghorns and blood samples were taken at intervals for test. The cells were tested with a 1: 5 dilution of a stock virus which was consistently active to 1: 120 throughout the series of tests. Table 3 shows the development of agglutinability with increasing age.

The strongly reacting cells of the 14 day group did not give an agglutination end point above 1: 40 but cells from the same chickens at 28 days gave, in nearly every case, the same end point (1: 120) as was obtained with adult fowls of known susceptibility.

TABLE 3.

Development of agglutinability of red cells by vaccinia virus in chickens.

Age of chick.	Number tested.	Number with strongly positive agglutination. +++	Number with weakly positive agglutination. + or ±	Number with completely negative agglutination. −
Unhatched	50	0	0	50 (100%)
2 days	27	0	2 (7%)	25 (93%)
14 days	23	6 (26%)	3 (13%)	14 (61%)
28 days	16	7 (44%)	3 (19%)	6 (37%)
6–30 months	78	43 (55%)		35 (45%)

DISCUSSION.

The wide applicability of the Hirst reaction to influenza work makes it important to look for similar methods that can be used for other viruses. Our experience with vaccinia virus which can only be used effectively with cells from about half the individuals of a single species (and those individuals above a certain age) makes it likely that there are other viruses with restricted activity. Only by studying a wide range of species are cells suitable for given viruses likely to be found.

SUMMARY.

The range of species giving red cells agglutinated by influenza, Newcastle disease and vaccinia virus is shown in tabular form.

The influenza and Newcastle disease viruses show general resemblances in their agglutinative behaviour but can be divided into three groups comprising respectively (a) the three influenza A strains, (b) influenza B and (c) Newcastle disease.

Of the species tested only fowl, guinea-pig and human cells are likely to be of practical value for work with these viruses.

Vaccinia virus agglutinates one group of fowl cells to high titre but has only a low grade action on any of the other species of red cell tested.

About 50 p.c. of fowls (6 months old or over) give red cells agglutinable by vaccinia virus. This appears to be a genetic characteristic unrelated to age, sex or breed.

Embryonic red cells are all inagglutinable by vaccinia virus; agglutinability develops in those chicks which inherit the characteristic in from two to four weeks after hatching.

Acknowledgments. We wish to express our thanks to Mr. J. Warren, poultry-farmer of Ferntree Gully, from whom we obtained blood samples of a large series of fowls, and to Mr. W. C. McLister, poultry-farmer of Spotswood, who supplied us with the day-old cockerels.

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THE INCUBATION PERIOD OF THE EGGS OF *HALOTYDEUS DESTRUCTOR* TUCKER (ACARINA) AT DIFFERENT TEMPERATURES

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INTRODUCTION.

Halotydeus destructor is a sap-sucking mite of the Family Penthaleidae. In South Australia it is available in the field, in suitable numbers for experimental purposes, only during a portion of the wet season. During the past seven years one of us (D.C.S.) has carried out experiments on the incubation period of the eggs at different temperatures. He was appointed in September, 1942, as entomologist to the Royal Australian Air Force, and the data obtained on these experiments had not been analysed at that time. In view of the uncertainty as to when an opportunity would arise to do this work the data were left with the senior author, who analysed them in order to show the relationship between temperature and rate of embryonic development in this species. The methods of analysis employed are similar to those discussed in an earlier paper (Davidson, 1942).

EXPERIMENTAL TECHNIQUE.

Eggs were obtained from adult mites collected in the field as required. The mites were confined in small oviposition chambers, each chamber consisting of a shallow cylinder of terra-cotta earthenware, 6 in. in diameter and 3 in. deep. The lower end of the cylinder was pressed into damp sand in a tray to give a good seal; the upper end was ground flat and covered with a close-fitting sheet of glass. In this way the mites were retained in an atmosphere of high humidity which is necessary for oviposition.

Pieces of moistened filter paper were placed on the floor of each cage; the mites oviposited on the under side of the paper. They would not lay on dry paper. The filter paper was removed after eight hours and if eggs had not been laid in that time the mites were discarded: in some cases many thousands of eggs were laid in this period. The eggs used in the hatching experiments were therefore derived from a large number of mites, and they were less than twelve hours old when placed in the incubator.

Five batches, each batch consisting of one hundred eggs, were used in each test, except in Experiment Nos. 5, 6 and 14 in which 400, 400 and 300 eggs respectively, were used (Table 1). Each batch of eggs was arranged in the subdivisions of a 10 × 10 mm. grid ruled in pencil on the filter paper. A single egg was placed in each square and the grid was marked so that it could be orientated the same way for each examination. Eggs were manipulated with a fine sable hair artist's brush.

Eggs develop only when in contact with free moisture. Satisfactory moisture conditions during an experiment were maintained by placing a layer of damp sand in a petri dish and covering it with a disc of damp blotting paper. The piece of filter paper on which the eggs were arranged was placed on the blotting paper. The lid of the petri dish was lined with a disc of blotting paper, which was also kept moist.

The particular temperatures at which the eggs were incubated were maintained constant within a variation of 0·1° C. The eggs were examined under a dissecting microscope at twelve-hourly intervals and the number of hatched eggs was recorded. The weighted mean time for all the eggs which hatched at a given temperature was taken as the time required for the eggs to complete development at that temperature. It was calculated by adding the products of the number of eggs hatched in each 12-hourly inspection, and the time for development, and then dividing by the total number of eggs hatched. These values were used in determining the curves shown in Fig. 1.

ANALYSIS OF THE DATA.

The results of 21 experiments are available. They are given in Table 1.

The range of the incubation period is relatively long at some temperatures, but the distribution curve showing the number of eggs hatching on different days was of the normal type, with the peak number of hatched eggs occurring about the middle of the range. With the exception of the experiments at 27·5° C., approximately 90 p.c. of the eggs hatched in each experiment.

TABLE 1.

Showing results of experiments with eggs of H. destructor *incubated at the given temperatures. The mean incubation period, in each case, is the weighted mean of all the eggs which hatched. The period of incubation is defined as the time taken, after oviposition, for the eggs to develop completely and hatch.*

Expt. No.	Temp. °C.	Eggs hatched (p.c.).	Incubation period. Range in days.	Mean.
1	7·8	92	10·0–24·5	17·97
2	7·8	87	9·0–23·0	17·05
3	10·0	94	7·5–17·5	12·70
4	10·0	94	6·5–16·5	12·18
5	10·5	94	8·0–15·0	12·20
6	13	96	5·0–12·5	8·69
7	13	89	5·0–13·5	8·40
8	15	96	2·8– 9·5	6·87
9	16	97	3·8– 8·3	6·23
10	19	94	2·5– 7·0	4·75
11	20	96	2·5– 6·0	4·24
12	20	98	2·5– 6·0	3·96
13	21	96	2·5– 5·5	4·28
14	21	95	1·5– 5·5	4·08
15	21	94	2·0– 6·0	3·87
16	22	96	2·0– 6·0	3·66
17	23	91	1·5– 5·5	3·70
18	23	93	1·5– 5·5	3·86
19	24	91	2·0– 6·5	3·88
20	25	89	2·5– 5·5	4·09
21	27·5	51	1·5– 5·5	4·24

The observed mean incubation periods at 13 different temperatures are given in Table 2: the incubation periods for experiments at the same temperatures, given in Table 1, have been combined in this table. The marked drop in the percentage number of eggs which hatched at 27·5° C. is evidence of the harmful effects of heat on the developing embryos. Therefore the results of this experiment have been omitted in Table 2. The figures in column 3 of Table 2 represent the average proportion of total development achieved by the embryo per day at the given temperature: it is expressed as a percentage of the time taken for the embryo to develop completely at that temperature. In Fig. 1 the observed values for "y" and $\frac{100}{y}$ given in Table 2 are plotted against the appropriate temperatures.

TABLE 2.

Showing the average incubation periods for the eggs of H. destructor *(y); also the average percentage development of the embryo per day* $\left(\frac{100}{y}\right)$ *at the given temperatures.*

Temp. °C. (x).	Incubation period in days. (y).	Reciprocal × 100 $\left(\frac{100}{y}\right)$	Log. K $= \frac{100}{y} / \frac{100}{y}$
7·8	17·51	5·717	0·8365
10·0	12·44	8·065	0·6602
10·5	12·20	8·197	0·6517
13·0	8·55	11·720	0·4526
15·0	6·87	14·560	0·3196
16·0	6·23	16·050	0·2553
19·0	4·75	21·050	0·0551
20·0	4·10	24·390	$\bar{1}$·9258
21·0	4·08	24·510	$\bar{1}$·9212
22·0	3·66	27·320	$\bar{1}$·8098
23·0	3·78	26·490	$\bar{1}$·8423
24·0	3·88	25·770	$\bar{1}$·8716
25·0	4·09	24·450	$\bar{1}$·9232

The values for $\frac{100}{y}$ describe a sigmoid curve which exhibits a marked change in direction about 22° C. This may be considered as the "peak" temperature (Davidson, 1942). The lowest temperature at which the eggs can develop to completion and hatch was not determined, but 92 p.c. of the eggs hatched at 7·8° C, so that it may be expected that the value is much lower than this. The upper limit of temperature at which the eggs can develop to completion and hatch, was not determined: at 27·5° C. only 51 p.c. of the eggs hatched, so that it may be expected that the value is not much higher than this.

The observed values of $\frac{100}{y}$ at the ten temperatures from 7·8 to 22° C. (Table 2) fit a curve having the formula

$$\frac{100}{y} = \frac{K}{1 + e^{a-bx}}$$

This formula represents the average speed of development of the eggs per day $\left(\frac{100}{y}\right)$ at any given temperature (x) within this range. The computed values for the constants are: K = 44·95: a = 3·193706: b = ·16389. Therefore the formula for the temperature-velocity curve for the development of the eggs of *H. destructor* is

$$\frac{100}{y} = \frac{44 \cdot 95}{1 + e^{3 \cdot 193706 - \cdot 16389x}}$$

The curve shown in Fig. 1 has been computed from this formula.

The temperature-time curve is:

$$y = \frac{1 + e^{3 \cdot 193706 - \cdot 16389x}}{\cdot 4495}.$$

The curve shown in Fig. 1 has been computed from this formula.[1]

The temperature-velocity curve and the temperature-time curve illustrate faithfully the trend in the rate of development of the eggs between temperatures 7·8 to 22° C. This is well shown in Table 3. The observed values at the given temperatures are a good fit to the calculated curves. With values for $\frac{100}{y}$, the greatest deviation of any of the observed points from the calculated curve is ·971 at 20° C.; this is 4·1 p.c. of the computed value at this temperature. The root-mean square deviation for the ten observed points is ·4468, which is 1·9 p.c. of the calculated value at 20° C.

TABLE 3.

Showing the difference between the observed and calculated values for y and $\frac{100}{y}$ at the given temperatures. The calculated values were computed by means of the formulae for the curves in Fig. 1.

	Incubation period in days. (y)		P.c. development per day. $\left(\frac{100}{y}\right)$	
Temp. °C.	Obs.	Calc.	Obs.	Calc.
7·8	17·51	17·33	5·717	5·771
10·0	12·44	12·76	8·065	7·839
10·5	12·20	11·93	8·197	8·384
13·0	8·54	8·67	11·720	11·539
15·0	6·87	6·87	14·560	14·565
16·0	6·23	6·16	16·050	16·222
19·0	4·75	4·63	21·050	21·578
20·0	4·10	4·27	24·390	23·419
21·0	4·08	3·96	24·510	25·248
22·0	3·66	3·70	27·320	27·039

[1] The value of K = 44·95 was calculated from values for $\frac{100}{y}$ at 7·8, 14·8 and 21·8° C. The values for log K $- \frac{100}{y} \Big/ \frac{100}{y}$ were computed for each of the ten temperatures between 7·8 and 22° C. given in Table 2: these values were fitted to a straight line by the method of least squares by means of which the values of the constants a and b were obtained. The reader is referred to an earlier paper (Davidson, 1942) for further details relating to the curves described in this paper.

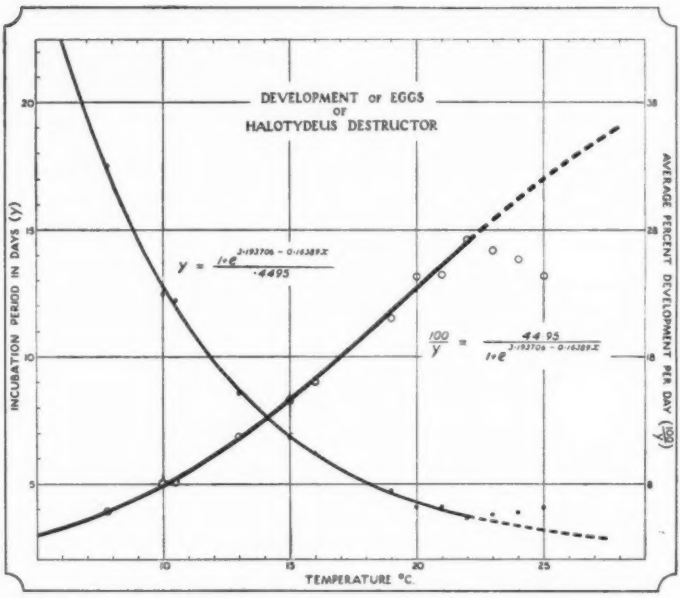

Fig. 1. Showing the distribution of the values for "y" (closed circles) and for $\frac{100}{y}$ (open circles) at the temperatures given in Table 2. The calculated temperature-time curve and the calculated temperature-velocity curve for the incubation period of the eggs of *H. destructor* are also shown.

SUMMARY.

The results are given of 21 experiments on the incubation period of the eggs of the mite *Halotydeus destructor* Tucker at 13 different, constant temperatures in the range 7·8 to 27·5° C. A total of 10,100 eggs were used, and about 90 p.c. of the eggs hatched in each experiment, except at 27·5° C., when only 51 p.c. hatched. The data have been fitted to a temperature-velocity curve and a temperature-time curve. A temperature-velocity curve having the formula:

$$\frac{100}{y} = \frac{44{\cdot}95}{1 + e^{3{\cdot}193706 - {\cdot}16389x}}$$

faithfully represents the trend in the speed of development of the eggs up to 22° C. (peak temperature). The greatest deviation of any of the ten observed points, within this range, from the calculated value, is ·971 at 20° C. The root-mean square deviation between the observed and calculated points for the ten given temperatures is ·4468 which is 1·9 p.c. of the calculated value at 20° C.

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GERMINATING SEEDS AS A SOURCE OF VITAMIN C IN HUMAN NUTRITION

1. ASCORBIC AND DEHYDROASCORBIC ACID CONTENTS OF SEVERAL VARIETIES OF SEEDS GERMINATING UNDER STANDARD CONDITIONS FOR VARYING PERIODS OF TIME.

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It is apparent, from Curtis's (1807) reference to Young's (1782) studies, that for upwards of one and a half centuries the value of germinating seeds as anti-scorbutic articles of diet has been known more or less widely to Western people, but there is evidence that their effectiveness was previously recognized and utilized in ancient China and throughout the ancient Indian Empire.

Strictly biological methods of estimating the anti-scorbutic potencies were employed in the earlier systematic work with germinating seeds—e.g. Fürst (1912), Chick and Hume (1917), Wiltshire (1918), Chick and Delf (1919), Delf (1921), Wats and Eyles (1932–3), Wats and Woodhouse (1933–4).

Few attempts have been made by chemical methods to estimate the "total" ascorbic acid content of germinating seeds, and inasmuch as the anti-scorbutic potency, or vitamin C content, of a material is believed to be represented by its content of ascorbic and dehydroascorbic acids, most of the chemical analyses have been insufficiently informative from the standpoint of nutrition; see, for example, Harris and Ray (1933), Johnson (1933), Ray (1934), Bogart and Hughes (1935), Reid (1938). Furthermore, some of these workers have doubted the reliability of the methods they have used for estimating ascorbic acid.

There has been considerable discordance in the findings of those who have worked with seeds. Thus the effect of exposure to light noticed by Reid (1938), was not found by Virtanen and Eerola (1936) who concluded that light was without effect upon the production of ascorbic acid in germinating peas. Again, Bogart and Hughes (1935) found a much lower concentration of ascorbic acid in germinating oats than did Harris and Ray (1933), and incidentally they could detect no difference in effect between light and dark conditions in seven days germination.

This article deals with certain preliminary work in a study aimed at determining those conditions of use of germinating seeds, which would be most satisfactory from the following points of view: (a) adequacy of the source of supply of seeds, (b) keeping qualities of the dry seeds, (c) viability, (d) concentration of the antiscorbutic substances, (e) ease of germinating and processing, the idea being to reduce equipment, time and labour to the barest minimum, (f) palatability of the processed material.

In the preliminary work here described, attention was directed mainly to the points (a), (c) and (d) above, and a brief survey was made of the values of the seeds available in quantity. This was done by germinating the seeds under arbitrarily standardized conditions and performing estimations upon the seedlings at various stages of growth. The seeds examined were wheat (*Triticum sativum*), navy beans (*Phaseolus vulgaris*), haricot beans (*Phaseolus vulgaris*), blue boiler or blue Prussian peas (*Pisum sativum*), and green gram (*Phaseolus mungo*).

EXPERIMENTAL.

Germination. The seeds were immersed in distilled water for 12–18 hours and then spread out on either wet purified cotton-wool or wet sand in shallow trays and covered with thin butter-muslin which was kept moist by capillary action. The butter-muslin was dispensed with as soon as the seedling roots had properly penetrated into the sand or cotton-wool. The air temperature

was about 25° C. during the daylight hours and about 20° C. during the night. The temperature of the wet cotton-wool and the seeds was generally a few degrees below that of the air. The germinating seeds were exposed to the diffused daylight of the laboratory, corresponding with 3 foot candles of illumination on the surface of the trays for about 13 hours of each 24 hours, and the butter-muslin excluded very little of this light.

Analyses. Moisture determinations were made by heating samples of the materials in air at 105° C. for 18 hours, and then exposing in a vacuum desiccafor over sulphuric acid.

Ascorbic and dehydroascorbic acid estimations were made by the methods described by Lugg (1942). Almost invariably, the six titrations recommended (A_1, A_2, A_3, B_1, B_2, B_3) were performed. Frequently also the $A_{\frac{1}{2}}$ titrations were made, but they were rarely greater than the A_2, and then only by very small amounts. There were indications that only very small quantities of extraneous reducers could have been present in the solutions which were titrated for the estimation of ascorbic acid (A_1 was about 5–10 p.c. greater than A_2, and A_3 was very small). Larger, but still fairly small, quantities of extraneous reducers were usually encountered in the solutions titrated for the estimation of "total" ascorbic acid (B_1 was about 10–25 p.c. greater than B_2, and B_3 was very small).

RESULTS AND DISCUSSION.

Until germination had commenced seeds had to be taken indiscriminately for analyses. After germination had commenced only germinating seeds were taken. Incidentally, there was no easily discernible correlation between size and viability of seeds.

The germinated pulse seeds all provided considerably higher concentrations of "total" ascorbic acid than did the wheat. This conforms with Fürst's (1912) generalization concerning pulse and cereal seeds. Clearly the germinating wheat had to be regarded as a very inferior source of vitamin C. Although the concentration of the vitamin in the wheat seedlings was increasing as growth proceeded, the material was becoming increasingly fibrous and less acceptable as food.

The haricot beans and navy beans were disappointing sources of vitamin C. Viability was not high and as most of the ungerminated seeds putrefied, the mixture of germinated and ungerminated material could not properly be used for food. With navy beans the concentration of "total" ascorbic acid was at no stage greater than 0·23 mg. per gm. of tissue, and this is rather low. The single experiment made to test the distribution of vitamin C in navy bean seedlings is of interest in showing that the concentration of vitamin C was substantially the same in the descending axes as in the cotyledons and minute plumules.

The remaining sources of vitamin C (blue-boiler peas and green gram) were satisfactory as regards viability and production of the vitamin. It will be seen that the green gram germinated very rapidly, and under the conditions of this work the stage of growth for maximum concentration of "total" ascorbic acid must have been rather critically defined. Although by comparison the blue boiler peas suffered from the disadvantage that they germinated and grew less rapidly there was no obvious peak concentration of "total" ascorbic acid at any stage of growth, and this is distinctly advantageous when the conditions under which the seeds are to be sprouted, for food, cannot be closely controlled. These and other considerations, particularly following the discovery that the bulk of green gram seeds was contaminated with eggs of *Bruchus chinensis*[1], led to the decision to confine the rest of the investigation to blue boiler peas. It is interesting to recall that Young (1782) referring to the antiscorbutic values of various germinated seeds, "gives the preference for gram, an East Indian grain, chiefly used in the feeding of horses".

From some of the earlier biological work with germinating seeds, it is possible to arrive at estimates of the vitamin C contents of the materials by comparing them with lemon juice and assuming a value for the lemon juice. Thus, using the estimations selected by Booher, Hartzler and Hewtson (1942), a mean for many samples of fresh juice is 0·4 mg. ascorbic acid per gm., and it is believed that fresh lemon juice contains very little dehydroascorbic acid.

[1] Kindly identified by Mr. H. G. Andrewartha.

TABLE 1.

Seed.	Age of seed (yr.).	Viability.*	Period germinating from time of moistening seeds (hr.).	Fresh weight of 100 seeds or plants (gm.).	Dry weight of 100 seeds or plants (gm.).	Stage of growth. Av. length (cm.) of: a: ascending axes d: descending axes.	Mg. ascorbic acid per gm. of fresh plant tissue.	Mg. "total" ascorbic acid† per gm. of fresh plant tissue.	Mg. "total" ascorbic acid† per gm. of original air-dry seed.
Wheat (*T. sativum*) "Sepoy" variety	1·1		0	4·0					
"			90	9·3			0·07	0·13	0·30
Wheat (*T. sativum*), mixed varieties, S.A., f.a.q.	0·2	98	0	3·84	3·61		0·00	0·04	0·04
			24	4·81			0·00	0·04	0·05
"			48	5·96		d 0·2	0·02	0·05	0·08
"			72	6·92		a 0·2, d 1·0	0·02	0·06	0·11
"			96	8·77	3·08	a 1·0, d 2·5	0·05	0·08	0·18
"			120	9·26		a 2·0, d 3·0	0·07	0·13	0·31
Haricot beans (*P. vulgaris*)	0·8 (?)	70	0	23·2	20·9				
"			30	50			0·06		
Navy beans (*P. vulgaris*)	0·8	75	0	28·0	25·3		0·00	0·04	0·04
"			24					0·10	
"			48	58·3		d 0·6	0·10	0·18	0·37
"			72			d 1·0	0·12	0·20	
"			96			d 2·5		0·22	
"			120	73·4		d 5·0	0·14	0·23	0·60
"			144	96·6		d 9·5	0·12	0·20	0·69
"			168	137·5		d15·0	0·13	0·21	1·03
" [descending axes only]			144	40·0		d 9·5	0·14	0·22	
Blue boiler peas (*P. sativum*)	1·0	99	0	20·7	18·4		0·01	0·05	0·05
"			24	37·3	17·6			0·07	0·13
"			48	40·5	17·0	d 1·0	0·15	0·22	0·38
"			72	47·7		a 0·5, d 2·5	0·24	0·32	0·74
"			96	54·0		a 1·2, d 3·5	0·26	0·32	0·84
"			120	57·3		a 3·0, d 4·0	0·24	0·35	0·97
Green gram (*P. mungo*)	>0·1	98	0	4·8					
"			24	12·0		d 0·1	0·11	0·15	0·38
"			48	14·0		d 1·2	0·31	0·38	1·10
"			72	17·0		d 2·5	0·22	0·28	1·00
"			96	19·7		d 5·0	0·17	0·25	1·03

* P.c. germination after 96 hours. † I.e. ascorbic acid plus dehydroascorbic acid calculated as ascorbic acid.

For example, Wiltshire (1918) germinated haricot beans for a total period of 72 hours at about 18° C. presumably in virtual darkness, and cooked the material gently (10 minutes in boiling water) to render it palatable to human scurvy patients. From his comparison of the material with lemon juice, it seems that 1 gm. of air-dry seed provided cooked, germinated material containing about 0·4 mg. "total" ascorbic acid.

Chick and Delf (1919) compared lemon juice, as a vitamin C source for guinea-pigs, with peas (*Pisum sativum*) and lentils (*Lens esculenta*) germinated for a total period of about 72 hours, the radicles then being about 1 cm. long. Their results suggest that the material germinated in light or in darkness contained only about 0·06 mg. of "total" ascorbic acid per gm.

Wats and Eyles (1932), using guinea-pigs in biological tests of the germinated seeds of various pulses, found green gram to be the best source of vitamin C. Assuming that their animals required 0·6 mg. ascorbic acid daily to escape scurvy, it would appear that the green gram germinated for a total period of 48 hours at 12°–24° C. and exposed to the diffused light of the laboratory during the daytime, probably contained rather more than 0·2 mg. "total" ascorbic acid per gm. of fresh material.

With the possible exception of the haricot beans germinated by Wiltshire (1918), the vitamin C production of germinating pulses in this earlier work appears to have been rather inferior to the production encountered in the present work. Comparisons are not easily made, however, because lemon juice is a rather indefinite standard and the stages of growth were often insufficiently characterized in this earlier work; and if the absorption of the vitamin from different foodstuffs is incomplete the degree of absorption may vary with the foodstuff.

On the other hand, Harris and Ray (1933), using a simple 2, 6-dichlorphenolindophenol titration of acid extracts, reported values for germinated seed peas which are considerably higher than the greatest shown in Table 1. However Reid (1938), who used a similar method of estimation, and Ihuzi (1940), have reported values respectively, for the cow pea (Vigna sinensis) germinated in light, and for germinated green peas, which are more nearly comparable with those shown for the blue boiler pea in Table 1.

SUMMARY.

Of seeds which were available in large quantities, namely, wheat (*T. sativum*), haricot beans (*P. vulgaris*), navy beans (*P. vulgaris*), blue boiler peas (*P. sativum*) and green gram (*P. mungo*) only the two last named were satisfactory as regards high viability and production of much vitamin C on germination. Their seedlings contained 0·35–0·38 mg. of "total" ascorbic acid per gm. of tissue after germinating at 20°–25° C. for a few days at a light intensity of 3 ft. candles during daylight hours (some 13 hours per day).

Acknowledgments. Thanks are due to Miss C. M. Eardley, Dr. I. F. Phipps, Mr. E. W. Pritchard and Mr. A. T. Pugsley for the identification of seeds.

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"NERVOUS DISTEMPER" IN DOGS

A PATHOLOGICAL AND EXPERIMENTAL STUDY, WITH SOME REFERENCE TO DEMYELINATING DISEASES IN GENERAL

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During the winters of 1941 and 1942 canine distemper occurred frequently in dogs and caused a heavy mortality among ferrets in Adelaide. At various intervals after the initial illness, and sometimes without such illness, nervous symptoms developed in many dogs; at autopsy we found in the brain demyelinating lesions similar to those first described by Pedrau and Pugh (1930). Our earlier studies of these lesions suggested the need for collateral experimental work. The combined observations led us to conclusions different from those reached by Perdrau and Pugh regarding the rôle of the virus of distemper in the genesis of the nervous lesions.

Cerletti (1912), Kantorowicz and Lewy (1922–3), Gallego (1928), Perdrau and Pugh (1930), Posrednik (1931), Marinesco, Draganesco and Stroesco (1933), Frauchiger and Walthard (1935), King (1939) and others have described the histological appearances in the nervous form of canine distemper. Although probably observed by others of these writers, demyelination was mentioned specifically only by Perdrau and Pugh, Posrednik, Marinesco, Draganesco and Stroesco, and King; at the time of publication of some of the papers interest in the demyelinating diseases was less widespread than it is to-day.

While Cerletti upheld the essential unity of all forms of the disease, Frauchiger and Walthard considered that the variability of the histological picture did not permit a statement upon the unity or otherwise of nervous and ordinary distemper. Kantorowicz (1933) reproduced all forms of the disease with material obtained from any clinical type. Puntoni (1923) and others used the brain as a source of distemper virus, and Robin and Vechiu (1926) isolated virus from the cerebro-spinal fluid. Perdrau and Pugh, on the other hand, doubted the identity of the demyelinating encephalomyelitis with distemper, since in their cases the temporal relation of one to the other was so variable and the latter did not appear even to be an essential antecedent. It should be added that a number of cases of "nervous distemper" have been complicated by, or confused with, protozoal infections [Kantorowicz and Lewy, Perdrau and Pugh, and Peters and Yamagawa (1936)]. Marinesco *et al.* regarded the nervous condition as a "*leuco-polio-névraxite*" intermediate between the diseases destroying mainly the grey matter (poliomyelitis, rabies, etc.) and the post-vaccinal type of encephalomyelitis which affects predominantly the white matter.

HISTOPATHOLOGICAL OBSERVATIONS.

We examined the brains of 4 dogs affected during the epidemic of 1941 and 5 during that of 1942. Eight of the animals had suffered from the systemic disease from 2 to 3 or, once, 6 weeks before developing nervous symptoms which ended fatally in from 3 to 7 days. In the case of one dog we elicited no history of previous distemper. Their ages ranged up to 4 years. The lesions in all were remarkably uniform in type and in distribution; in several cases the sections could not have been distinguished one from another without knowledge of their identity. The most severe lesions occurred constantly in certain parts of the cerebellum, at the periphery of the brain-stem, and in superficial regions around the interpeduncular fossa; in distribution they were thus almost as stereotyped as are those of poliomyelitis. They affected the white more than the grey matter. As will be seen later

[1] Work carried out by the authors with a grant from the National Health and Medical Research Council.

there was evidence that individual lesions were progressive and that in a particular case all were not necessarily of the same age.

In most parts of the nervous system meningeal infiltration was rarely pronounced, except where lesions occurred immediately beneath the pia, or where heavily infiltrated vessels emerged from the deeper tissues. In the former cases the meninges were often thickened by proliferation of their intrinsic cells. The infiltrating elements included lymphocytes, plasma-cells and rare polymorphonuclear leucocytes. Meningitis was usually more severe over the base of the cerebrum and over the cerebellum and brain-stem than elsewhere.

Areas of diffuse or more localized infiltration of the nervous tissue with polymorphic microglial cells occurred in the cerebral cortex, but were numerous in only three cases. Vessels in their neighbourhood were heavily cuffed with cells similar to those in the meninges; sometimes these cells overflowed into the nervous tissues. In spite of the duration of many of the lesions, presumed from the experimental results (q.v.), polymorphonuclear leucocytes were not infrequent; they occurred in the nervous tissues rather than in the perivascular exudates. Occasional nerve-cells were replaced by neuronophagic nodules. In general the vascular changes took one or two forms, of which either might be more evident in a given vessel or in the vessels of a particular animal (see Cerletti, 1912). Some vessels showed perivascular cuffing as described above, others considerable proliferation of their adventitial cells with increase of reticulum fibres and a general thickening of their walls. From the smaller vessels proliferated adventitial cells appeared to pass out as phagocytes into the surrounding tissues. When the capillaries and pre-capillaries showed this thickening over a circumscribed or more diffuse area, the vascularity of the part was greatly emphasized (Cerletti). In other cases the vascular lumina seemed to be constricted, or a complicated mass of cells showed a very inconspicuous lumen. Complete occlusion of vessels by thrombosis or other mechanism was not seen.

These microglial foci with their attendant vascular changes resembled those found in many virus diseases. In two cases they were numerous in the vertical cortex about midway between the frontal and occipital poles, in one case in the cortex forming the ventral lip of the *sulcus rhinicus anterior* and in the whole subpial zone between this and the base of the brain. In other cases they were less numerous and found almost exclusively in the molecular layer of various cortical areas. On occasion they occurred also in the basal ganglia, especially in the caudate nucleus near the wall of the ventricle, in the cornu Ammonis, and in the interior of the brain-stem and spinal cord. In them the myelin sheaths were often missing. Rarely we observed areas of complete focal necrosis of some standing.

The more constant, severe and extensive lesions occurred at other sites, namely at the base of the brain around the interpeduncular fossa, around part or the whole of the circumference of the brain-stem, and in the white matter of the cerebellum. They nearly always attained their maximum intensity in the roof, floor and lateral angles of the fourth ventricle, especially in the medullary vela, and in the cerebellum where the white matter of the folia was especially affected (Fig. 1). Other areas particularly selected by the pathological process were the surface layers of the optic tracts, fascia dentata, crura cerebri and infundibulum. A few parts of the walls of the lateral and third ventricles were similarly but much less affected, as were the nerve-roots emerging from the brain-stem and areas of the white matter at the periphery of the spinal cord, especially of the posterior columns.

Where these lesions were most severe the tissues had undergone partial or almost complete disintegration. Granular corpuscles were often numerous and were sometimes almost the only cells present. In other places the neuroglia was progressively altered, and very large cells with abundant protoplasm and multiple nuclei were frequent. The myelin sheaths were ballooned, fragmented or absent. The axis-cylinders also suffered considerable damage, and in the most severely affected areas were wholly wanting; for the most part, however, though showing degenerative phenomena they were less affected than were the myelin sheaths. [This opinion is expressed with full knowledge of the contentions of Alexander and Putnam (1940)]. The vessels showed the changes described above, and in severely affected areas reticulum fibrils extended widely into the surrounding tissues (Fig. 2). In the dog dying 6 weeks after distemper the areas of demyelination contained numerous collagen fibres. Lymphocytes, plasma-cells and sometimes a few polymorphonuclears were present in the tissues.

In what appeared to be the most recently affected parts of the white matter, the tissues presented a spongy appearance from ballooning of the myelin sheaths. The glial cells were enlarged and contained inclusions (see below), the oligodendroglial nuclei were pyknotic, rare polymorphonuclear leucocytes were present; there was little or no vascular change or microglial activity, and the axis-cylinders were well-preserved. Sometimes too around a severely demyelinated area with much increase of cells these milder changes were evident, suggesting perhaps an extension of the pathological process.

Various observations point to these appearances as representing successive stages of one and the same morbid process, and not as King (1939) suggests different kinds of degeneration of the myelin. In the "spongy" areas there were few or no products of myelin-disintegration. That other lesions were of comparatively recent development was shown by the absence of scharlach-

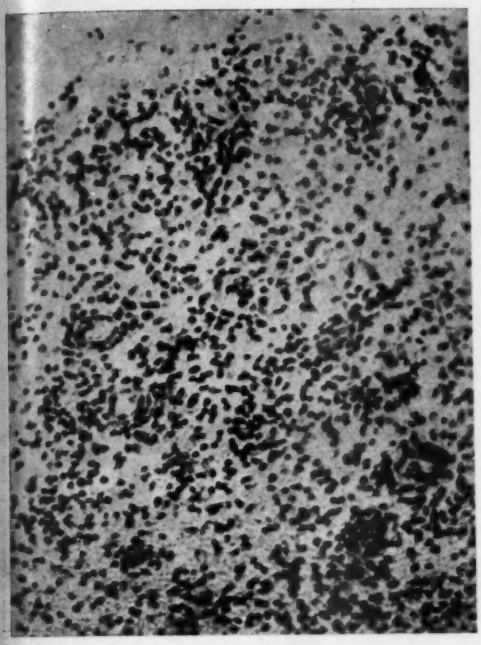

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 1. Nervous distemper. Low power view of the cerebellum showing the intense cellular infiltration of the white matter of a folium, with considerable destruction of the granular layer. Iron-alum-haematoxylin and van Gieson.

Fig. 2. Nervous distemper. In a severely damaged area of the cerebellum there is increase of reticulum in a vessel wall and spreading of reticulum fibrils through the affected nervous tissue. Hortega's method.

Fig. 3. Dog 4. Advanced demyelination around a group of vessels in the spinal cord. Weil's stain.

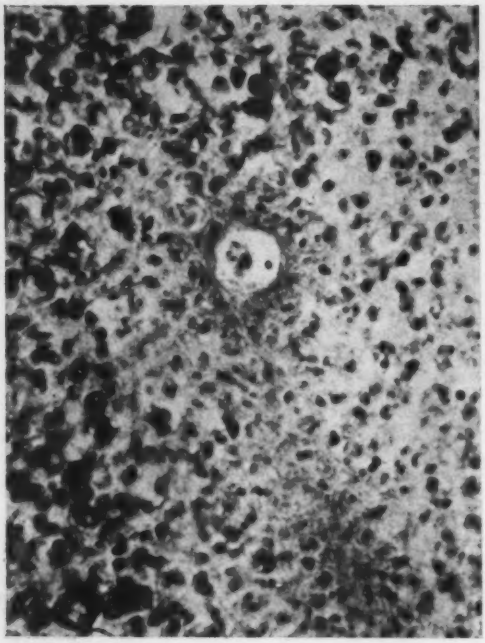

Fig. 4.

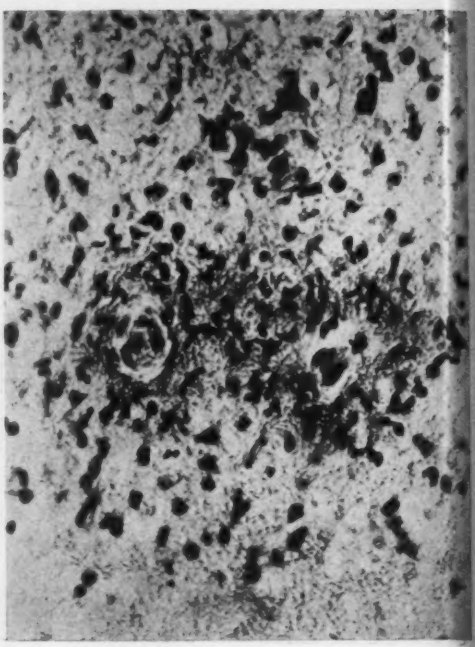

Fig. 5.

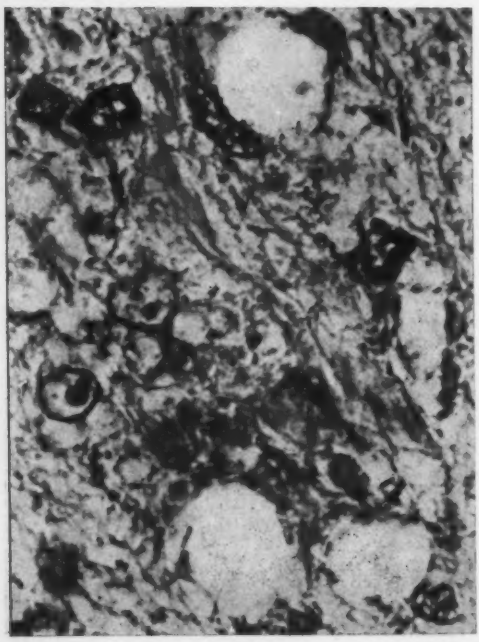

Fig. 6.

Fig. 4. Dog 4. More recent demyelination in the spinal cord. The darker area immediately around the vessel indicates a zone of perivascular necrosis and infiltration with fibrin. Weil's stain.

Fig. 5. Dog 4. Vessels similar to those in Fig. 4. The areas of perivascular change are infiltrated with altered microglial cells. Iron-alum-haematoxylin and van Gieson.

Fig. 6. Area of early demyelination in the anterior medullary velum of a dog infected with nervous distemper virus and dying without showing nervous symptoms. The tissues are vacuolated from ballooning or loss of myelin sheaths. The glial cells are enlarged and two contain definite acidophilic nuclear inclusions Phloxin-methylene blue.

staining material and anisotropic needles in any quantity; on the other hand, the phagocytic cells contained much material staining by Weil's method. In the most advanced lesions neutral fat was present. Again in several cases the lesions were obviously not all of the same age. Thus one dog with advanced destruction of myelin in the cerebellum showed in the cerebral cortex a focus characteristic of recent involvement by a neurotropic virus; many of the nerve-cells contained inclusions and exhibited early degenerative phenomena, while scanty polymorphonuclears infiltrated the tissues and a few mononuclears occupied the perivascular spaces. The glial cells also contained inclusions, while the oliogodendroglial nuclei were pyknotic.

The lesions in both grey and white matter were sometimes clearly perivascular, but by no means always so. As Perdrau and Pugh (1930) pointed out, the larger lesions surround groups of vessels rather than a single vessel; the perfectly concentric demyelination characteristic of post-vaccinal encephalitis is not a feature of the present disease.

As already mentioned, in the cerebellum the central white matter of the folia was almost always most affected. When the granular layer suffered it was affected almost always from below; sometimes the deeper lesions burst through into the overlying molecular zone. Occasionally the cerebellum appeared to be involved from the surface; the Purkinje cells were destroyed and the granular layer attacked from above. In the granular layer it was by no means uncommon to see clusters of minute pyknotic nuclei numbering perhaps 30 or 40 lying in the closest juxtaposition. This appearance could have resulted if a single cell had undergone very considerable mitosis before degenerating.

When the optic tracts were affected, they suffered patchy demyelination from the surface and not in the centre as is the case in poisoning with KCN and NaN_3 (Hurst, 1940, 1942).

The white matter of the cerebral hemispheres was severely affected in only one case; it showed the vascular changes described above together with glial enlargement and microglial proliferation most pronounced in their neighbourhood. There was little degeneration of the myelin sheaths or axis-cylinders.

In some affected areas the nerve-cells showed chromatolysis and resembled those in a recovering case of simian poliomyelitis killed about a month after the acute injury. Mostly, however, they were quite well-preserved, even though the surrounding tissues were severely affected.

In all cases but two[2] (and in one of these the paraffin sections did not include areas of change), acidophilic intranuclear inclusions were present, though ordinarily they were not very abundant. Resembling small herpetic inclusions of the more deeply staining and not obviously granular type, they differed appreciably from the smaller intranuclear and intracytoplasmic bodies present in the bronchial epithelium, bile-ducts, etc., in known distemper (see de Monbreun, 1937; Green and Evans, 1939; Watson and Plummer, 1942). As will be seen later, similar inclusions were found in the nervous system after experimental infection with distemper. They occurred in neuroglial but not in oligodendroglial cells; in the former an inclusion might occupy one of several nuclei. They also occurred rarely in nerve-cells. Some Purkinje cells thus affected showed margination of the nuclear chromatin, but otherwise seemed normal with intact protoplasm and tigroid substance. Ependymal cells covering a lesion abutting on the ventricular system had often proliferated and contained inclusions, in these cases both in the nucleus and in the cytoplasm; sometimes those immediately over the lesion had desquamated and their neighbours showed inclusions. Only once was an inclusion seen in what appeared to be an adventitial cell. Inclusions were usually present in the spongy lesions of the white matter. They were often fairly numerous in those of intermediate age and absent from those in which most of the normal structures had been destroyed, possibly because of lack of a suitable type of cell. Their distribution was erratic, however; of two otherwise identical lesions only one might show inclusions.

From the foregoing it appears probable that the lesions of nervous distemper in dogs constitute a progressive encephalopathy; both the individual lesions and those in the animal as a whole are progressive. The presence of intranuclear inclusion bodies suggests that the virus is still active several weeks after the initial infection. The earliest histological changes in the white matter are degeneration of the myelin-sheaths, enlargement of the neuroglial cells, pyknosis of the oligodendroglial nuclei, and perhaps sparse polymorphonuclear infiltration; the other changes described above follow later. Apart from the presence of inclusions, many of the lesions bear a close resemblance to those in some of the more severe demyelinating diseases of man. Associated with them are changes in the grey matter which correspond with those found in many virus diseases of man and animals. The term used by Marinesco, Draganesco and Stroesco—"*leuco-polio-névraxite*"—adequately summarizes the nature of the pathological process; their description of the lesions tallies very closely with ours.

[2] Neither of these was the case without history of distemper.

ATTEMPTS AT TRANSMISSION.

Attempts at transmission followed naturally upon the discovery of intranuclear inclusions in the demyelinated areas. We infected dogs and ferrets with bacteriologically sterile suspensions of some of the brains and spinal cords described above; survivors were tested for immunity to known distemper virus kindly supplied by Dr. F. W. Morgan of the Commonwealth Serum Laboratories. Other animals were inoculated with known distemper virus and the survivors tested with material from cases of nervous distemper. In retrospect it seems likely that some of the immunity tests were performed before the initial infection had run its full course; nevertheless the experiments show that the cases of nervous distemper harboured a virus of behaviour identical with that of distemper. Table 1 summarizes the clinical and post-mortem data of many of these experiments.

Distemper is notoriously prone to spread among the inmates of an animal house, and we had none of the elaborate facilities for isolation or the highly-trained animal attendants available to Dunkin and Laidlaw (1926a and b). We performed our experimental work somewhat tediously over a considerable period of time, using rooms at opposite ends of the animal house alternately for new animals and for those under test for immunity. Only one infective agent was in use at one time and we ensured complete decontamination between successive groups of inoculations. Using ferrets and very young puppies during inter-epidemic periods, we were satisfied that the results described below were truly positive. In the epidemic period of 1942, however, our whole stock of ferrets in use for experiments with influenza succumbed to distemper introduced by unknown agency; this incident exemplifies the difficulties inseparable from work with distemper in an ordinary animal house. A further difficulty is the lability of the virus of distemper which does not survive long in the cold-room; by maintaining our material at —20° C. we prolonged considerably the period over which it was infective.

Results.

Inoculations with material from cases of nervous distemper. Dogs 3 (IC), 10 (IM) and 13 (IC + IM) developed fever or other symptoms but recovered; subsequently they survived administration of Commonwealth Serum Laboratories distemper virus intramuscularly or intracerebrally and intramuscularly.

Dog 9 (IC) gave a febrile response and was killed when suffering from severe nervous symptoms on the 8th day. In the white matter of the cerebrum and cerebellum and in the brain-stem foci of microglial proliferation were associated with perivascular cuffing and productive changes in the vessel walls. The cerebellar changes did not involve the white matter of the folia. No inclusions were detected.

Dog 15 (IC + IM) suffered from fever, anorexia and catarrhal symptoms; it died with pneumonia on the 12th day. The white matter of the cerebellar folia contained a few small microglial foci in which the myelin sheaths were ballooned and distorted. In the anterior medullary velum early myelin disintegration accompanied clear-cut glial enlargement and degenerative phenomena in the oligodendroglial nuclei (Fig. 6). Nuclear inclusions identical with those in the spontaneous disease were present. Intranuclear inclusions resembling those of distemper were present in cells of the Malpighian bodies of the spleen, and intranuclear and intracytoplasmic inclusions in the bronchial epithelium

Dog 14 (IC + IM) was febrile on the 15th day. On the 19th day it vomited; it was vicious and obviously ill. After apparent recovery it received on the 24th day C.S.L. distemper virus intracerebrally and intramuscularly. Fresh fever ensued on the 34th–36th days and the animal died suddenly in status epilepticus on the 36th day. Lesions in the central nervous system resembled closely those in nervous distemper. They were much more advanced than those in Dog 15, or those found at a roughly comparable stage in animals receiving C.S.L. virus (see below). For this reason it seems possible that they may have resulted from the earlier injection, and that the immunity test was performed in an animal already destined to develop nervous distemper. The lesions consisted in demyelination, etc., at the periphery of the brain-stem and in the folia of the cerebellum. Intranuclear inclusions were present. No distemper-like inclusions were seen in most of the viscera, but the liver showed many areas of acute necrosis with acidophilic inclusions of Cowdry's type A and much larger than those seen in distemper. This observation is of interest since it recalls the findings in the puppy constituting Group 3 of de Monbreun's dogs (1937): Green and Evans (1939) assumed that this animal was suffering from a mixed infection with distemper and an unknown virus.

Ferret 17 (IC + IM) responded with slight fever; it developed fresh fever following intracerebral and intramuscular inoculation of C.S.L. distemper virus, but recovered within a few days.

TABLE 1.

Transmission experiments during 1941.

Known distemper virus (canine spleen) from Commonwealth Serum Laboratories.

GROUP B.

Dog 4 (IM). Three separate samples C.S.L. virus on 1st, 11th and 24th days. On 46th day nervous distemper (I.C.). Fever from 52nd–57th day. On 57th day paralysis of hind limbs. Killed.

Dog 5 (IM). As preceding. Febrile reaction on 52nd–54th day following test inoculation of nervous distemper (IM).

Dog 7 (IM). C.S.L. virus (third sample in two preceding). Fever from 3rd–7th day. Death 7th day.

Ferret 14 (IM). C.S.L. virus used for testing Dog 10. Irregular temperature from 5th day onwards. Death 21st day.

GROUP F.

Dog 16 (IC + IM). Fever 3rd and 7th days. Fits, salivation 7th day. Killed.

Dog 17 (IC + IM). Fever 3rd day. Nervous distemper (IC) 17th day. No reaction.

Dog 18 (IC + IM). Fever 2nd, 3rd and 6th days. Nervous distemper (IC) 17th day. Fever 25th day.

Dog 19 (IC + IM). Fever 3rd and 9th days. Nervous distemper 17th day. No reaction.

Dog 20 (IC + IM). Fever 3rd day. Severe vomiting 7th day. Killed.

Ferret 19 (IC + IM). Febrile reaction from 5th–13th day. Then irregular temperature. Became gradually weaker and killed when moribund on 39th day.

Ferret 20 (IC + IM). Fever 3rd and 6th days. Very ill with generalized muscular twitching 9th day. Killed.

Ferret 21 (IC + IM). Irregular fever from 6th day on. Very ill with twitchings and convulsions 23rd day. Killed.

Ferret 22 (IC + IM). As preceding. Killed 23rd day.

Ferret 23 (IC + IM). Irregular temperature throughout. Died 39th day with enormous spleen.

GROUP G.

Dog 23 (IC + IM). Fever, possibly biphasic, from 5th day on. Shivering, cough 10th day. Progressively worse. Death 17th day. Pneumonia.

Dog 24. (IC + IM). No definite reaction.

Ferret 24 (IC + IM). Fever 13th day. Killed 14th day with haemorrhagic lung and pleural exudate.

Ferret 25 (IC + IM). Fever 7th and 12–13th days. Killed.

Brains from local cases of nervous distemper.

GROUP A.

Dog 3 (IC). Sharp febrile reaction from 3rd–7th day. No response to 3 separate samples C.S.L. virus (IM) on 18th, 29th and 53rd days; last sample killed Dog 7.

Dog 9 (IC). Sharp febrile reaction from 5th–8th day. Fits and salivation 8th day. Killed. Spleen greatly enlarged.

Dog 10 (IM). Slight fever from 5th–8th day. No response to C.S.L. virus (IM) 15 days later; this virus killed Ferret 14.

GROUP C.

Dog 13 (IC + IM). Tremor, doubtful fever, anorexia, cough 13th day. Recovery. No reaction to C.S.L. virus (IC + IM) 24th day.

Ferret 15 (IC + IM). Fever from 9th–13th days. Died with subnormal temperature 16th day.

GROUP D.

Dog 14 (IC + IM) Fever 15th day. Sick, vomiting, vicious 19th day. C.S.L. virus (IC + IM) 24th day. Fresh fever 34th–36th days. Sudden death with epilepsy 36th day.

Ferret 16 (IC +IM). Febrile reaction from 15th–17th day. Thereafter irregular temperature and death on 40th day with pneumonia, pericarditis, etc.

GROUP E.

Dog 15 (IC + IM). Fever, catarrhal symptoms, anorexia. Death with pneumonia 12th day.

Ferret 17 (IC + IM). Slight fever from 6th–8th day. C.S.L. virus (IC + IM) 24th day. Fever from 27th–31st days. Recovery.

IC = intracerebral inoculation. IM = intramuscular inoculation.

The experiments in Group A were performed with material from a single dog. The immunity tests were done with the C.S.L. virus used in the experiments in Group B. The nervous distemper virus used for Group B was that employed for the initial infection in Group A. The experiments in Groups C, D and E were performed with material from three separate brains. These were pooled for the immunity tests in the animals of Group F. The C.S.L. virus used for Group F served to test immunity of Groups C, D and E.

Ferrets 15 (IC + IM) and 16 (IC + IM) died after 16 and 40 days respectively. Few changes were present in single sections of the nervous system. In Ferret 15 the main lesions were in the lungs, where the bronchial epithelium exhibited inclusions indistinguishable from those in distemper. Ferret 16 showed widespread lesions with similar inclusions in the lungs, trachea, bile-ducts and spleen.

Inoculations with known distemper virus. Dog 24 (IC + IM) suffered no reaction; it was not tested further. Dogs 5 (IM), 17 (IC + IM), 18 (IC + IM) and 19 (IC + IM) remained free from symptoms or showed some fever; tested later intramuscularly, or intracerebrally and intramuscularly, with material from cases of nervous distemper, they failed to respond or showed a febrile reaction. This reaction was marked only in Dog 5.

Dogs 7 (IM), 16 (IC + IM), 20 (IC + IM) and 23 (IC + IM) reacted with fever and other symptoms; they died or were killed when very ill after 7 or 17 days. Of Dog 7 we examined only the spleen; it showed in the Malpighian bodies masses of pyknotic and fragmented nuclei. Dog 16 exhibited nervous symptoms before death. At the lateral angles of the pons and in the floor of the fourth ventricle the glial cells were enlarged and many of their nuclei contained inclusions identical with those found in the cases of nervous distemper. The oligodendroglial nuclei were pyknotic. The myelin sheaths were undergoing fragmentation. At this stage the microglia was almost inactive. Dogs 20 and 23, and two others in another set of experiments, presented no definite nervous symptoms; they showed in the brains only occasional perivascular infiltration and rare microglial foci without inclusion bodies. In none of this group of animals were inclusions detected in the viscera.

Dog 4 tolerated without obvious reaction 3 intramuscular injections of C.S.L. virus; the virus used on the third occasion at least was virulent for other animals inoculated at the same time (see Table 1). Thereafter the animal received intracerebrally material from a case of nervous distemper. Six days later it developed fever and 11 days later paralysis of the hind limbs. As in the case of Dog 14, but to a much greater degree, the lesions in its nervous system were more advanced than would have been expected had they resulted from the last intracerebral injection. In addition they were atypical in distribution and showed cellular and vascular changes not observed in any other case either of distemper or of nervous distemper.

The main lesions consisted in large and extremely cellular "plaques" in the brain and spinal cord. While situated almost wholly in the white matter, they did not always respect the boundaries of adjacent grey matter, and cellular infiltration and vascular changes extended in places into the cerebral cortex or posterior horns of the spinal cord. The centres of some of these plaques consisted solely of blood vessels and granular corpuscles; in others the picture was that of complete demyelination (Fig. 3) with infiltration and proliferation of a number of types of cell. Nearer the edges perivascular areas of demyelination, broader and less sharply outlined than those in post-vaccinal encephalitis, were fusing to enlarge the demyelinated areas; in intervening strips of tissue the myelin sheaths were ballooned and fragmented, but the axis-cylinders were relatively normal. The areas were much more densely cellular than those in any other dog, so that they were very conspicuous to the naked eye; the polymorphic microglia or granular corpuscles contained fragmented myelin or scharlach-staining substances according to the stage of destruction. The larger vessels were heavily cuffed, and numerous lymphocytes overflowed into the nervous tissues; some polymorphonuclears also were present. The vessels showed considerable adventitial proliferation, and the capillaries marked swelling of their endothelium. Other vessels at the periphery of the areas were surrounded by zones of necrosis (Figs. 4 and 5), which with the perivascular spaces were impregnated with fibrin as in acute haemorrhagic leucoencephalitis (Hurst, 1941); a zone of partial demyelination often encircled the necrotic area. Small haemorrhages were frequent. In the cerbral cortex, brain-stem, etc., microglial foci and perivascular cuffing existed as in ordinary cases of nervous distemper. The folia of the cerebellum, surface layers of the brain-stem, cerebral peduncles, etc., did not show the lesions characteristic of that condition. No inclusions were seen in the brain or viscera.

Ferrets 14 (IM) and 19–25 (IC + IM) died or were killed when very ill 9–39 days after inoculation of C.S.L. virus. The symptoms were typical of distemper, and in the viscera the characteristic inclusions were present in one or other of the sites commonly affected (see Green and Evans, 1939; Watson and Plummer, 1942). Only single sections of the nervous system were examined; in but two animals did these show lesions. In the cornu Ammonis of ferret 21 many nerve-cells were necrotic; others contained acidophilic or slightly basophilic inclusions resembling more closely those produced by hypertonic saline solutions (Lee, 1936, and others) than do most inclusions due to viruses. Ferret 19, which had survived for 39 days, had suffered considerable destruction of the nerve-cells in the cornu Ammonis, with marked compensatory gliosis; nuclear inclusions were present in both nerve and glial cells. The walls of the vessels were the seat of productive changes, and areas of perivascular and focal demyelination occurred in the affected regions. To the best of our knowledge this is the first occasion on which demyelination has been observed in the ferret. It should be added that in several other ferrets the section did not include the cornu Ammonis, which is a structure often selected for attack by neurotropic viruses (see Hurst, 1934).

Effect of Hyperimmune Anti-distemper Serum on the Pathogenicity of Brains of Cases of Nervous Distemper.

Five ferrets each received intramuscularly 2 c.c. of a centrifuged 10 p.c. brain-suspension from various cases of nervous distemper. An equal number of controls received the same dose of brain-suspension mixed with 5 c.c. anti-distemper serum (C.S.L.); before inoculation the mixture was allowed to stand for 1 hour at room temperature. The control animals remained free from symptoms. The others developed symptoms characteristic of distemper; 4 showed typical inclusions in the viscera. The disease produced in animals receiving no serum was transmitted to other ferrets with similar results.

Comment.

With bacteriologically sterile brain and cord from cases of "nervous distemper", we produced in dogs and in ferrets symptoms and pathological changes identical with those due to known distemper virus. In both infections inclusion bodies occurred in the same situations and were morphologically indistinguishable. We did not detect them in all animals infected with either "nervous distemper" or distemper; the most careful work has failed to reveal them in every case of distemper (Green and Evans, 1939; Watson and Plummer, 1942). We received the impression that, in the general viscera, inclusions were found more readily in ferrets than in dogs, and when the infection ran a longer rather than a shorter course. In most cells of the brain inclusions were purely intranuclear. In the ependyma, however, as in the respiratory mucosa, bile-ducts, etc., they were both intranuclear and intracytoplasmic. The inclusions in the brains of animals inoculated with known distemper virus were morphologically identical with those in naturally-occurring "nervous distemper".

The cross-immunity tests between distemper and the pathogenic agent present in the brains in cases of "nervous distemper" were less satisfactory than we hoped. If our interpretation is correct, however, the results are compatible with the belief that we were dealing with two viruses closely related, if not antigenic duplicates. Leaving out of consideration for the moment the cases of Dogs 4 and 14, they show that animals previously infected with either agent suffer no ill-effects or at most a febrile reaction from introduction of the other. The dog, of course, often resists inoculation of distemper virus, a fact which speaks both for and against our interpretation. Where dogs resisted known distemper virus following inoculation with material from cases of "nervous distemper", the low virulence of distemper virus for the dog, and not immunity, might be held responsible. On the other hand, tests in the opposite direction show at least that the virus in cases of "nervous distemper" is of similarly low pathogenicity. The result in Ferret 17 is perhaps more significant. Distemper in the ferret is a much more serious illness than in the dog and nearly always ends fatally (Dunkin and Laidlaw, 1926a); yet Ferret 17 after recovering from infection with "nervous distemper" resisted distemper, whereas all other ferrets in these and later experiments succumbed. Finally, anti-distemper serum in large doses neutralizes the pathogenic agent present in cases of "nervous distemper" in dogs. Taking these observations in conjunction with the pathological and clinical findings in naturally and experimentally infected animals, there seems little doubt that distemper virus was present in the brains of animals suffering from "nervous distemper". It may be recalled that most of the cases of nervous disease followed distemper after intervals of from 2 to 6 weeks, but that in one animal there was no history of preceding distemper.

We regard the observations made on the brains of Dogs 15 and 16 as most important. In these animals, infected intracerebrally and intramuscularly with the brain of a case of "nervous distemper" and with C.S.L. distemper virus respectively, there existed already at the time of onset of symptoms of distemper lesions

in the nervous system characteristic of "nervous distemper". Though of very restricted distribution in the roof or floor of the 4th ventricle, folia of the cerebellum and lateral part of the pons, they pointed to an origin of the nervous lesions at the time of the initial infection. They suggested very strongly, therefore, that demyelination results directly from the action of the specific virus, and that it is not due to some other factor operative after infection with distemper. Thus the disseminated encephalomyelitis following distemper cannot be compared with that following vaccinia, etc., in which no satisfactory evidence has yet been adduced to show that the vaccinia or other virus of the initial exanthem is directly responsible for the encephalopathy. In this conclusion we differ from Perdrau and Pugh. Our experiments did not explain why in the natural disease the nervous lesions progress when the systemic infection ends; that they do progress and that fresh acute lesions may be present in the nervous system some weeks after clinical distemper has been shown in the first section of this paper. One possibility is that antibodies are developed in low concentration or that they fail to penetrate the blood-brain barrier; in equine encephalomyelitis the nervous system may be infected successfully in animals recovered from the systemic infection (Hurst, 1936a).

The findings in Dogs 4 and 14 still await consideration. At first thought the latter might be held to disprove the existence of the cross-immunity accepted above. As we have mentioned, however, the lesions in the nervous system seemed too advanced to be due to the test inoculation of nervous distemper. Accepting the thesis that the foundations of nervous distemper are laid during the early stages of infection, it is inevitable that some animals tested for immunity within a few weeks of the initial infection would already be suffering from the latent brain-disease. We regard Dog 14 as a probable case in point.

In Dog 4 also the lesions almost certainly antedated the test for immunity, but here they were distinctly different in type and distribution from those seen in any other case. Some writers have claimed that allergy is the exciting factor in post-vaccinal encephalomyelitis, though no experimental evidence is forthcoming to support this belief. Whether or not the unusual manifestations in this case were due partly to some kind of allergic action can only be determined by further experiment.

DISCUSSION.

Some authors (Perdrau and Pugh, 1930; King, 1939) have regarded as uncertain the relation of the demyelinating encephalopathy of dogs to dog-distemper; the former suggested an analogy to post-vaccinal encephalomyelitis in man. In the present paper we have shown that:

(a) a virus indistinguishable from that causing distemper is present in the brains of dogs so affected, together with morphological changes similar to those produced by known distemper virus;

(b) known distemper virus may provoke lesions identical with those in nervous distemper.

Why dogs surviving the acute systemic phases of distemper should later develop nervous lesions is not fully explained, unless by the two following observations. During the systemic phase of the malady following intracerebral and intramuscular inoculation, even in the absence of nervous symptoms, we have detected early lesions in the regions later affected in nervous distemper. Secondly, Hurst (1936a) has shown that in a virus disease such as equine encephalomyelitis, recovery from the systemic malady is not necessarily followed by immunity of the central nervous system; apparently this is protected by the blood-brain barrier from antibodies circulating in the blood.

The only reasonable interpretation of the foregoing results is that the aetiological agent of nervous distemper (post-distemper encephalomyelitis) is the distemper virus itself. Animals surviving the immediate effects of a virus disease of the central nervous system usually show more or less destruction of tissue. Since most neurotropic viruses affect predominantly the nerve cells, lesions are localized chiefly in the grey matter; here the residual effects may range from complete destruction of tissue with the formation of porencephalic cavities, through glial-mesodermal scars, to a pure sclerosis resulting from the replacement of destroyed neurons by glial tissue (see Hurst, 1936b). In a few virus diseases the white matter also is involved to a variable extent, and demyelination results (e.g. Perdrau, 1936).

Apparently in dog-distemper the affinity of the virus for certain parts of the white matter is more marked than with most viruses affecting the nervous system. According to their severity the lesions may cause severe destruction in the white matter, which is reduced to a mass of granular corpuscles, blood-vessels and collagen, or they may be responsible for demyelination accompanied or not by destruction of the axis-cylinders. Though wholly different in their aetiology and distribution, these lesions are comparable with those produced by simple chemical agents such as KCN and NaN_3 (Hurst 1940, 1942), in which the intensity of the poisoning determines either total necrosis of the white matter or demyelination with or without relative preservation of the axons. To the same extent they are analogous with the lesions in acute haemorrhagic leucoencephalitis (Hurst, 1941), in which perivascular necroses (with or without haemorrhage) are represented by perivascular demyelination in the less severely affected areas.

In their wider implication the results discussed have a definite bearing on the problem of demyelination in general. We must believe that demyelination is not a specific process due to any one cause, but a type of response of the white matter to various noxious stimuli insufficient to cause full necrosis of tissue. It follows that aetiologically the demyelinating diseases do not constitute a homogeneous group, a view already envisaged by Innes and Shearer (1940).

CONCLUSIONS.

From the brains of dogs showing demyelination and suffering clinically from "nervous distemper", we have isolated a virus identical in its behaviour with that of distemper. The lesions tend to localize in certain restricted areas of the cerebellum and brain-stem; they are described fully in the present paper.

In fatal infections with known distemper virus, sometimes in the absence of nervous symptoms, early demyelinating lesions may be present in some of the areas commonly affected in "nervous distemper".

In both groups of cases the same type of inclusion-body may be present.

There is some evidence of cross-immunity between the agent of "nervous distemper" and distemper virus. Anti-distemper serum neutralizes the pathogenic agent present in cases of "nervous distemper".

We conclude that the cause of "nervous distemper" is the distemper virus itself, and that the demyelinating lesions produced by it represent damage to the white matter short of complete necrosis. Apparently this virus has a particular affinity for the cells of the white matter of the brain.

From this and previous work we conclude that demyelination is a type of reaction of the white matter, and that the demyelinating diseases as a whole are not variations of a single pathological process.

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ANTIBACTERIAL SUBSTANCES PRODUCED BY MOULDS

3. THE DETECTION AND ESTIMATION OF ANTIBACTERIAL ACTIVITY *IN VITRO*.

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When investigating the antibacterial activities of various moulds, the question arose as to which methods were most satisfactory for the detection and estimation of their activity. To answer this question I used a number of methods, both qualitative and quantitative, with moulds of Groups 1 and 2 (Atkinson, 1943a), each of which had a different range of antibacterial activity.

EXPERIMENTAL METHODS.

The following were qualitative tests:

(a) *The "cup" method* (Fleming, 1942; Atkinson, 1943b). The medium was serum-agar; the fluid to be tested was placed in a "cup" cut from the centre of the agar in a petri-dish. After drying, strokes from broth cultures of different organisms were arranged radiating from the cup. When the fluid in the cup was active, growth of the susceptible test-organisms did not occur close to it; the greater the activity of the antibacterial agent the greater the zone of inhibition.

(b) *Incorporation of the fluid in a culture-medium with subsequent inoculation of test-organisms.* The medium was nutrient (heart-muscle extract) agar; the fluid to be tested was added to melted agar at 50° C. to give a 1 in 10 dilution; the agar was then poured into a plate. After thorough drying the surface was inoculated with streaks of various organisms, with precautions to prevent spreading beyond the streak. After incubation at 37° C. for 24 hours the growth on the test-plate was compared with that on a control plate containing only nutrient agar.

For quantitative estimations of activity the following methods were tested:

(c) The "ring" method of Florey's school (Abraham *et al.*, 1941), modified by attaching the glass ring to the surface of the plate by running in a few drops of melted agar. The test organisms were *Staph. aureus* for Group 1 moulds and *Bact. typhosum* for Group 2 moulds.

(d) *The method of dilution in a solid medium.* Serial dilutions of the metabolism fluid were mixed with nutrient agar as in (b). The surface of the agar was stroked with different test-organisms and the growth compared with that on the control plate.

(e) *The method of dilution in a fluid medium.* Serial dilutions of the metabolism fluid in nutrient broth were inoculated with a standard loopful of an 18-hour broth-culture of the test-organism. A control tube of nutrient broth was inoculated at the same time for comparison with the test.

(f) *Test for germicidal activity.* A standard quantity of the test-organism was added to a given amount of each of a series of dilutions of metabolism fluid. After a certain time at a certain temperature, equal amounts of each dilution were inoculated into nutrient broth; this was incubated for 24 hours at 37° C. and was read by comparison with a control culture.

I observed the various precautions of technique recommended in the routine performance of disinfectant tests, e.g. Rideal-Walker test.

RESULTS.

Qualitative Tests.

(a) In the examination of 70 metabolism fluids for activity this method proved both satisfactory and sensitive. It gave good indication of range and degree of activity with *Staph. aureus*, *C. diphtheriae mitis*, *Strep. viridans*, *Bact. coli* and *Bact. typhosum*. Its advantage lay in its ability to test easily and quickly activity against a number of different bacteria. As serum-agar was used, a difference in extent of activity as estimated by this method and by others using only unenriched media appeared.

(b) This method also proved satisfactory and presented fewer technical difficulties than (a). The use of ordinary agar instead of serum-agar limited the range of test-organisms more than in (a), but with those organisms which grew satisfactorily similar results were obtained. However the results gave no indication of the degree of activity against a given organism, since no growth occurred with susceptible organisms; whereas with (a) the greater the distance between the cup and the bacterial growth the greater was the inhibitory power against the organism concerned. In spite of these disadvantages method (b) appeared to me to be the one of choice for preliminary qualitative tests because of the ease of its performance and the reliability of results.

Quantitative Tests.

The quantitative methods were tested with two different metabolism fluids, one containing penicillin produced by a penicillium No. 1 of Group 1 and the other containing penicidin produced by a penicillium No. 9 or No. 67 of Group 2. The results with No. 1 fluid are given first, those with No. 9 and No. 67 later.

Quantitative Tests with Penicillin Fluid.

(c) Florey and his collaborators recommended this method for the estimation of penicillin potency *in vitro*. They mentioned various considerations affecting its accuracy but considered it satisfactory for their work. The method consisted of the inoculation of the entire surface of an agar plate with a few drops of a broth culture of *Staph. aureus*; the plate was dried at 37° C. and a glass ring was sealed with agar to the centre of the surface of the agar in the plate. Florey attached his rings by means of an internal bevel on one end which was inserted into the agar. About 0·5 ml. of the metabolism fluid was placed in the ring and the plate incubated for 24 hours at 37° C. Around the ring the staphylococci failed to grow over an area varying in extent with the potency of the fluid. As Florey pointed out, the width of the zone of inhibition varied with a number of other factors.

In the present investigation I studied the influence of a number of these factors, (I) variation in depth of agar in the plate, (II) length of time of drying of the plate after inoculation with *Staph. aureus*, (III) size of the ring, (IV) concentration of penicillin in the fluid, (V) amount of fluid in the ring, (VI) time allowed for diffusion of fluid from the ring, (VII) age of test-organisms.

(I) With agar plates of from 1–12 mm. depth the zone of inhibition was largest on the 1 mm. plates and smallest on the 12 mm. plates. Florey and his colleagues measured the diameter of the zone and called this the "assay value" (which included the diameter of the ring). Obviously the area enclosed in the ring is exposed to a uniform and maximum effect of the inhibiting substance. A more precise estimate of the degree of inhibition is obtained by measuring only the zone outside the ring, that is, the distance between the outer edge of the ring and the line where bacterial growth begins. This value I refer to as the "zone size". It was determined by measuring the total diameter of the zone or "assay value", subtracting the external diameter of the ring (20 mm.), and dividing the result by two.

The following figures, the average of 16 readings taken 8 each from two duplicate plates, show the effect of varying the depth of the agar.

Depth of agar in mm.	Assay value.	Zone size.
1	41	11
2	40	10
4	38	9
8	37	9
12	36	8

The maximum clarity of the zone of inhibition was obtained with a depth of agar of 4 mm. With greater thickness the zone was less clear, while thinner layers dried up. In subsequent work I used a depth of 4 mm., and a quantity of a 24 hours broth culture of *Staph. aureus* just sufficient to cover the plate without making it excessively wet. The resulting growth was entire.

(II) Tests performed under similar conditions showed that drying the plates for various times at 37° C. before adding the penicillin fluid had no effect on the size of the zone of inhibition. In subsequent work the time of drying was 15 minutes.

Time of drying.	Assay value.	Zone size.
0	40	10
15 minutes	39	10
35 minutes	39	10

(III) The diameter of the ring affected the zone size as follows:

External diameter of ring in mm.	Zone size.
10·0	7
6·5	6
3·5	5

(IV) Florey and his collaborators pointed out that the assay value of a solution would only be a measure of its potency if the figure fell within a certain range, outside of which the assay value bore little relation to the concentration of penicillin. In my experiments the zone size also altered very little with dilution of the penicillin fluid up to a certain point, beyond which the change became roughly proportional over a small range of values round about 7 mm. In quantitative work therefore a comparison of the strengths of two solutions would be made by relating the dilution of each producing equal zone sizes of approximately 7 mm. The fluid used here was satisfactory at a dilution of 1/25.

(V) In these tests the penicillin fluid was diluted to produce a zone size of 7 mm. under the conditions of the previous experiment. Then different volumes were used in a set of ring tests with the following results:

Volume of penicillin fluid.	Assay value.	Zone size.
0·10 ml.	33	7
0·25 ml.	34	7
0·50 ml.	34	7
0·75 ml.	34	7

Provided that the volume lay between 0·25 and 0·75 ml. the size of the zone of inhibition was unaffected.

(VI) Since the zone size depends upon the distance through which the penicillin fluid diffuses into the medium, tests were made to determine the effect of preliminary diffusion. Penicillin fluid was placed in the rings on plates which previously had not been inoculated with *Staph. aureus*. After standing at 37° C. for various times, the plates were inoculated, dried and incubated in the usual way. Eight readings on two duplicate plates gave the following average figures:

Time interval before inoculation	Assay value.	Zone size.
0 (control)	37	9
2 hours	40	10
5 hours	44	12
24 hours	greater than 60	greater than 20

The figures show that the longer the period of diffusion before the addition of the staphylococci, the greater the zone size.

(VII) The age of staphylococci exposed to the action of penicillin affected materially the formation of a zone of inhibition. It was found that incubation for five hours or longer before the addition of the penicillin fluid to the ring, prevented the formation of any zone of inhibition of growth. The formation and size of this zone obviously depends upon the interaction of two opposing factors, (1) the diffusion of the penicillin fluid through the agar tending to produce a larger zone with the passage of time, and (2) the multiplication of staphylococci increasingly counteracting the formation of the zone up to five hours, after which macroscopic growth does not appear to be retarded. It is easy to imagine that this time may depend on technique, e.g. size and age of inoculum, strain of staphyloccocus, etc.

(d) A reliable quantitative method was that of dilution in nutrient agar. The antibacterial titre could be determined against several organisms at the same time on one set of plates. *Staph. aureus* and *Bact. typhosum* were successfully tested together using serial twofold dilutions of the penicillin fluid. With *Bact. typhosum* some attenuation of growth compared with that of the control was evident at 1 in 10 dilution, the most concentrated tested; *Staph. aureus* was completely inhibited at 1 in 800 but showed attenuated growth at 1 in 1,600. Closer dilutions between 1 in 800 and 1 in 1,600 were tested with *Staph. aureus*; a fairly sharp end-point appeared between 1 in 1,000 and 1 in 1,200. Thus this method was sensitive to small changes in concentration of the inhibitor, and the antibacterial titre was proportional to the dilution.

(e) With *Staph. aureus* titration by dilution in nutrient broth gave the same antibacterial titre of 1 in 800 and attenuated growth at 1 in 1,600. With closer dilutions no definite end-point could be read. Thus dilution in agar was a more useful method, not only because finer titrations could be performed but also because several titrations with different organisms were possible at the same time on the same plates, allowing a more exact comparison of results.

(f) As was expected this method was of little value for the estimation of the potency of the penicillin fluid which against *Staph. aureus* is mainly bacteriostatic. Five ml. of dilutions of the fluid in Ringer's solution or broth were mixed with one standard loopful of a staphylococcal broth culture 6 hours old. In broth at 37° C. bactericidal activity was detected in a dilution of 1/50 after 20 hours but not after 16 hours. Higher dilutions were not bactericidal. In Ringer's solution at 4° C. no bactericidal effect was detected with a dilution of 1/5 in 18 hours.

Quantitative Tests with Penicidin.

(c) As the penicidin fluid showed activity against both *Staph. aureus* and *Bact. typhosum*, two sets of ring plates were made. The staphylococcal plates showed only hazy ill-defined zones of inhibition but the typhoid plates showed very clear zones. In subsequent tests, therefore, *Bact. typhosum* was used. The dilution of the penicidin fluid was never proportional to the zone

size; with the more concentrated dilutions, however, greater alterations in zone size occurred with altered concentration than was the case with the penicillin fluid. For comparison of the relative strengths of two fluids they should be diluted to produce an equal zone size of about 6 mm. The fluid used here gave a satisfactory zone when diluted about 1/25. With cultures of *Bact. typhosum* up to 72 hours in age zones were produced in every case. With cultures younger than one hour the zones were quite free from growth. With older cultures what appeared to be an attenuated growth occupied the zone; this, however, did not grow in subculture.

(d) This method proved very satisfactory with penicidin fluid against *Bact coli*, *Bact. paratyphosum* B, *Bact. typhosum*, *Bact. typhi-murium*, *C. diphtheriae mitis*, haemolytic streptococcus and *Staph. aureus*. A dilution of 1/200 inhibited all the test organisms. A dilution of 1/400 inhibited *Bact. typhosum* and the haemolytic streptococcus; other organisms showed an attenuated growth with the exception of *Bact. coli* and *Staph. aureus*, which grew as well as in the controls. Good growth of *Bact. typhosum* and the haemolytic streptococcus occurred at 1/1,600.

(e) Titration by dilution in broth gave the same titres as (d) of 1/200 for *Staph. aureus* and 1/800 for *Bact. typhosum*.

(f) The germicidal activity of No. 67 mould metabolism solution (containing penicidin) was investigated in conjunction with *Bact. typhosum*. In broth at 37° C. the germicidal titre increased with time, being 1/10 at 4 hours, almost 1/20 at 6 hours and 1/40 at 24 hours. The antibacterial titre by broth titration (e) was also 1/40 with trace of inhibition at 1/80, showing that the lack of growth in the dilutions 1/40 and lower was due not to bacteriostatic but to bactericidal action. This constitutes a difference between penicidin and penicillin. In Ringer's solution at 4° C. no bacterial activity appeared in 1/2 dilutions in 23 hours, indicating that the rate of disinfection is greatly increased at 37° C. in spite of the presence of a nutrient medium (broth) encouraging multiplication of the test-organism.

DISCUSSION.

Both the cup method (a) and the agar incorporation method (b) for qualitative work appeared satisfactory. Method (b) involved less trouble than (a) and gave the same general results; it is recommended for preliminary detection of antibacterial activity.

For quantitative estimation of antibacterial activity the ring method (c) and two dilution methods, one using agar (d) and the other broth (e), were compared for two different types of metabolism fluid, one containing penicillin and the other penicidin. Variations in technique affect the ring method; among other things the depth of agar in the plate, the size of the ring, and the amount of fluid in the ring are important. The concentration of inhibitor in the fluid only influences the size of the zone of inhibition within certain close limits. To compare the potency of two penicillin fluids, they should be diluted to produce an equal zone size of about 7 mm. on *Staph. aureus* plates. With penicidin fluid a little more latitude may be allowed; a zone size of about 6 mm. on *Bact. typhosum* plates is recommended. The expression of the results as zone size removes the difficulty of including in the figure a constant representing the diameter of the ring and therefore is more satisfactory for tests relating concentration of inhibitor and size of zone of inhibition. The ring test is a useful method for following the production of antibacterial substance during early growth of a mould; one ring test for each culture each day readily indicates increase in concentration. However, for estimation of antibacterial activity the method of dilution in agar (d) is more satisfactory; the ring test, in which a standard technique is necessary, is too laborious. Dilution in broth (e) gave the same titres as (d) but is less economical, as separate sets of broth are needed for each test organism whereas the agar method (d) allowed several different species of bacteria to be tested under identical conditions on the same plate. It also shows up readily any contaminating organisms which may have gained access with the metabolism solution. (No contaminants were detected in this method (d) throughout this work.) The penicidin fluid is unlikely to become contaminated with bacteria as it is active against so wide a range of organisms. Thus one of the chief objections to the dilution method has been removed. The answer obtained, being a titre, stands in its own right and does not need to be related to

some other value as in the ring test. However it is well to titrate the test organisms with a standard solution from time to time to detect any alteration in susceptibility of the organisms concerned after many subcultures on artificial media.

The germicidal tests with penicidin fluid are of little value as an estimate of potency; the bactericidal titre depends, of course, on the conditions of temperature, time, diluting fluid, quantity of inoculum tested, etc. It is well recognized (Withell, 1942; Hobbs and Wilson, 1942) that methods of estimating bactericidal power by endeavouring to determine complete killing of added bacteria are unsatisfactory. They are open to severe criticism not only on account of the difficulty of determining with any certainty that all bacteria are dead, but also on account of the variability of resistance to disinfection of the individual cells of any given bacterial culture. To compare the bactericidal activity of two different penicidin preparations it would be better, as in the technique of the above workers, to compare the conditions necessary to produce a reduction of 50 p.c. in the bacterial population added.

In the concentrations used here the bactericidal tests were of little use for the penicillin fluid.

SUMMARY.

A number of methods for both qualitative and quantitative detection of antibacterial activity have been tested with a variety of active mould metabolism solutions. The relative merits of the various methods are discussed. For rapid detection of activity and for reliable estimation of potency of penicillin and penicidin fluids the method of dilution in agar is recommended. It allows the titration of the fluid against a range of bacteria under identical conditions.

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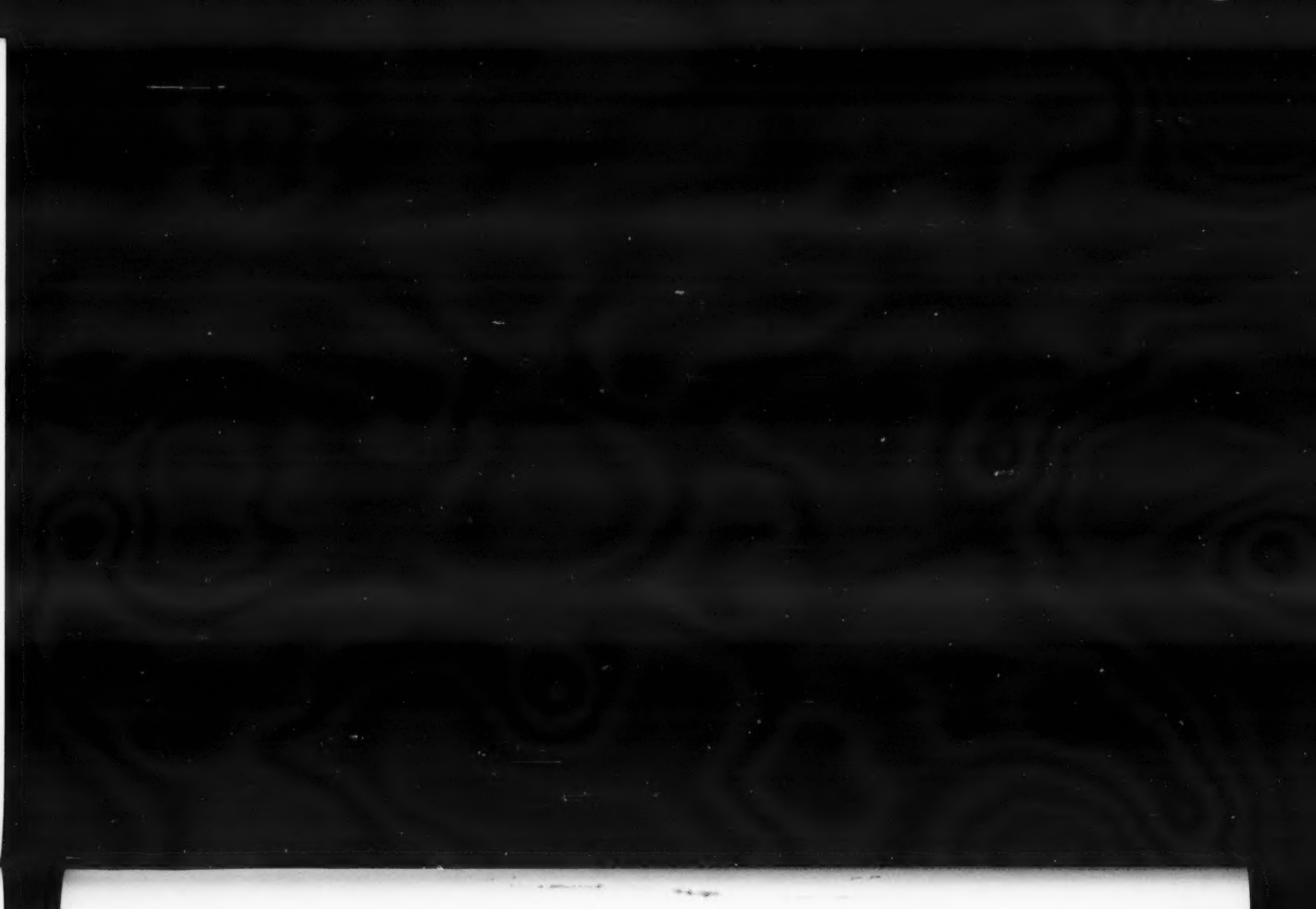
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